Role of intracellular transport, sorting and release of membrane type 1 matrix metalloproteinase (MT1-MMP) in tumor cell invasion and metastatic dissemination

Thesis

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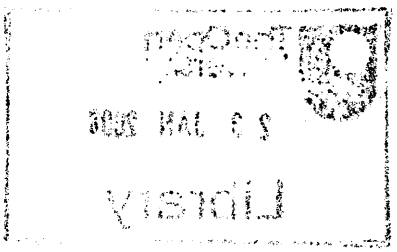
Role of Intracellular Transport, Sorting and Release of Membrane Type 1 Matrix Metalloproteinase (MT1-MMP) in Tumor Cell Invasion and Metastatic Dissemination



Marco Mazzone, M.S.

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Sponsoring Establishment: Consorzio Mario Negri Sud

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Abstract

The primary cause of death in cancer patients is the development of metastatic secondary tumours in distal organs. This is a complex process during which metastatic tumour cells need to overcome the natural barriers impeding access to vascular or lymphatic pathways: importantly they also need to alter the extracellular environment to allow ectopic cancer growth in distant locations. These events generally require the direct participation of released and exposed matrix metalloproteinases (MMPs), a family of related zinc-dependent proteases.

Regulation of MMP activity is known to occur by modulation of gene expression, via zymogen activation and inhibition by tissue inhibitors of metalloproteinases (TIMPs). It is now evident, however, that modulation of intracellular trafficking, membrane sorting and cell surface release can also regulate MMP activities. These latter topics are still not well understood and, for this reason, represent the main aims of the present study.

In particular, it has clearly emerged that MT1-MMP, a membrane-type MMP, is essential for the growth for human cancers, and acts by disrupting the three-dimensional matrix, which would otherwise impede cell proliferation. Ablation of the gene encoding MT1-MMP causes the most significant phenotype among the MMP knockout mice, and suggests that the activity of MT1-MMP cannot be substituted by any of the other members of the family. For this reason, the main focus of my PhD thesis is the regulation of MT1-MMP activity.

Here I show that, in a melanoma cell line, the majority (80%) of MT1-MMP is sorted to detergent-resistant membrane fractions; however, it is only the minor (20%) detergent-soluble fraction of MT1-MMP that undergoes intracellular

processing to the mature form. Also, this processed MT1-MMP is the sole form responsible for ECM degradation in vitro. Finally, furin-dependent processing of MT1-MMP is shown to occur intracellularly after exit from the Golgi apparatus and prior to arrival at the plasma membrane. It is thus proposed that the association of MT1-MMP with different membrane subdomains might be crucial in the control of its different activities: for instance in cell migration and invasion and other less defined roles such as MT1-MMP-dependent signaling pathways.

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Abbreviations

2D bidimensional

3D three dimensional

α1-PDX α1-antitrypsin Portland mutant

α1-PI α1-proteinase inhibitor

aa amino acids

ADAM adamalysin-related metalloproteinase

ADAMTS ADAM with thrombospondin type I motifs

AP adaptor protein

BFA brefeldin A

BM basement membrane

bp base pairs

BSA bovine serum albumin

cAMP cyclic adenosine monophosphate

CD cluster of differentiation

cDNA complementary DNA

Ci curie

CS calf serum

DDR discoidin domain tyrosine kinase receptors

DMEM Dulbecco's modified minimal essential medium

DNA deoxyribonucleic acid

DNAse deoxyribonuclease

DRM detergent-resistant membranes

EC_{50} 50% effective concentration

ECL enhanced chemiluminescence

ECM extracellular matrix

EGF epidermal growth factor

EM electron microscopy

ER endoplasmic reticulum

ERK extracellular signal-regulated kinase

FCS fetal calf serum

FGF fibroblast growth factor

g acceleration of gravity

GAG glycosaminoglycan

GF growth factor

GFP green fluorescence protein

GPI glysosyl phosphatidyl inositol

GST glutathione S-transferase

GTP guanosine 5'-triphosphate

GTPase guanosine triphosphatase

h hour

HA hemagglutinin

HT human type

IF immunofluorescence

IGF-BP insulin-like growth factor-binding protein

IL-1 interleukin-1

IP immunoprecipitation

kDa kilodalton

KO knockout

LB Luria Broth

LDLR low density lipoprotein receptor

LRP low-density lipoprotein receptor-related protein

mCD methyl-β-cyclodextrin

M6PR mannose 6 phosphate receptor

MEM modified minimal essential medium

MMP matrix metalloproteinases

MMR MT1-MMP polyclonal rabbit antiserum

mRNA messenger RNA

MT-MMP membrane-type matrix metalloproteinases

NC non collagenous

O.N. over-night

PAGE polyacrylamide gel electrophoresis

PAI plasminogen activator inhibitor

PBS phosphate buffered saline

PCR polymerase chain reaction

PG proteoglycan

PM plasma membrane

PMA tetradecanoyl-phorbol-acetate

Rab Ras-associated small GTPase

RECK revision-inducing cysteine-rich protein with kazal motifs

RGD Arg-Gly-Asp sequence

RNA ribonucleic acid

rpm revolutions per minute

RT room temperature

SPARC secreted protein, acidic and rich in cysteine

SDF stromal cell-derived factor

SDS sodium dodecyl sulfate

TCA trichloroacetic acid

TfR transferrin receptor

TGF transforming growth factor

TGN *trans*-Golgi network

TIMP tissue inhibitor of metalloproteinases

TNF tumor necrosis factor

TPA tetradecanoylphorbol-13-acetate

Ts temperature sensitive

TTSP type II transmembrane serine proteinase

U unit

uPA urokinase plasminogen activator

uPAR uPA receptor

VEGF vascular endothelial growth factor

VSV-G vesicular stomatitis virus G protein

Wt wild-type

CHAPTER 1

Introduction

Pericellular proteolysis and cell migration are pivotal events in development, vascular remodeling, wound healing, inflammatory responses, and cancer invasion. As part of these processes, different cell types need to traverse basement membrane barriers and move across interstitial, basement membrane, or temporary matrices. An invasive cell phenotype can be triggered by altered interactions of cellular receptors with extracellular matrix (ECM) components and soluble factors. Limited proteolysis by cell surface proteinases such as membrane-type matrix metalloproteinases (MT-MMPs) allows cells to make their way through the modified matrix. This movement implies continuous ECM attachments and detachments by adhesion receptors. Cell surface proteinases not only remodel ECM components, but also modify pericellular growth factors or their binding proteins and other cell surface proteins, including receptors or other proteolytic enzymes. Therefore, when MT-MMPs are targeted to the leading edge of migrating cells, proteolysis may not only clear the way for movement, but may also provide cells with directional cues by modifying the environment in the direction of movement.

In the introduction that follows, I will review in-depth the role MT-MMPs play in ECM remodeling. This presupposes a basic knowledge of ECM constituents. Hence, I will briefly describe its general structure and the main characteristics of its most important components, and also the molecules that matrix components use to interact with cells that make the matrix or are supported by it.

1.1 Extracellular matrix

The ECM consists of collagens, glycoproteins, proteoglycans, and glycosaminoglycans (Aumailley and Gayraud, 1998; Zagris, 2001). It is a highly organized fibrillar meshwork, which serves as substratum for cell adhesion and migration. ECM also constitutes a barrier that maintains tissue integrity, impedes cell migration, and regulates molecular diffusion and transfer of stimuli. In addition, ECM forms a dynamic cellular microenvironment, which plays an important role in the determination of cell phenotype (Boudreau and Bissell, 1998; Streuli, 1999). It mediates the passage of information to and from other cells directly through its components and by storing and modulating the function of growth factors/cytokines and other regulatory factors including processing enzymes and their inhibitors.

As I shall describe below, the specialized ECMs of interstitial connective tissues and basement membranes are two structurally and functionally distinct ECM categories (Fig. 1.1). The main features these two classes have in common is that their basic structure is defined by a collagen scaffold, although the collagens that make up the scaffold are quite different, as is their three-dimensional architecture (Bosman and Stamenkovic, 2003). Various adhesive glycoproteins, including laminins, and proteoglycans adhere to the scaffold and interact with the cells in, or adjacent to, the matrix. Interaction with these cells is through matrix receptors, of which the integrins constitute the most important class (for a review, Miranti and Brugge, 2002).

1.1.1 Interstitial matrix

Interstitial matrix surrounds, and is formed by, connective tissue cells such as fibroblasts, osteoblasts, chondrocytes, and macrophages. It consists of a meshwork of protein fibers embedded in an amorphous glycosaminoglycan/proteoglycan substance (Aumailley and Gayraud, 1998).

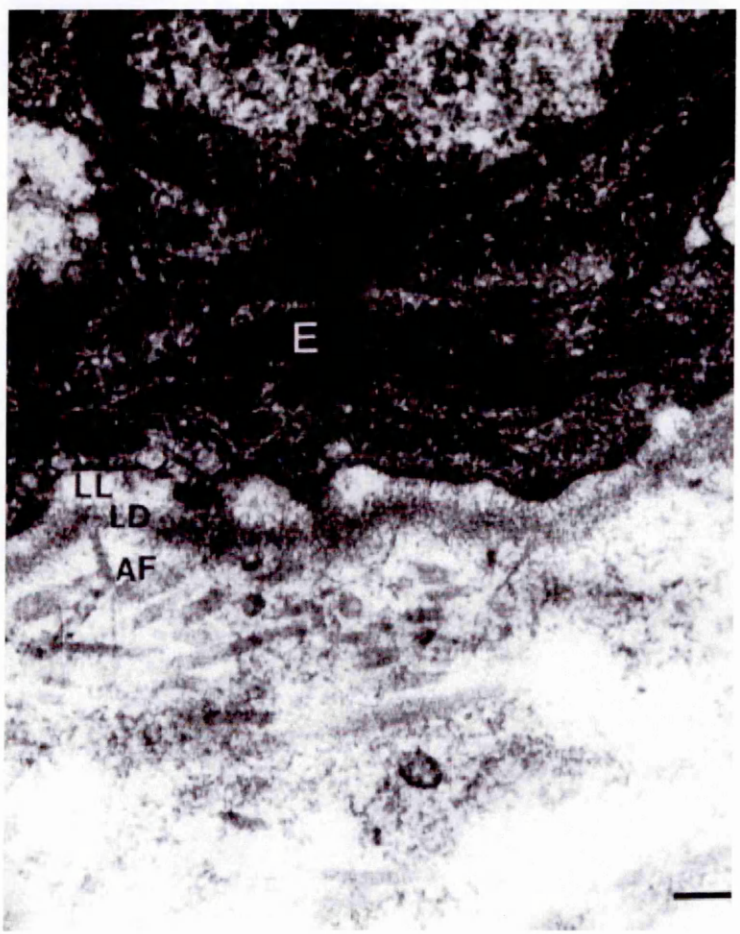


Fig. 1.1. Ultrastructure of the extracellular matrix (reproduced from Bosman and Stamenkovic, 2003). The basement membrane with its lamina lucida (LL) and lamina densa (LD) is found adjacent to an epithelial cell (E). The interstitial matrix contains collagen fibrils and is in close proximity to the basement membrane anchoring fibrils, composed of type VII collagen (scale bar 0.1 μM).

The composition and molecular architecture of matrices differs substantially among tissues like bone, cartilage, tendons, ligaments, dermis, vessel walls, and the stroma of parenchymal organs (i.e. liver, kidney, etc.).

Collagens are the most abundant structural components of interstitial ECM in all tissues (Aumailley and Gayraud, 1998; Prockop and Kivirikko, 1995) and are divided in two main groups (for a more detailed classification, see Table 1.1):

1) Fibrillar collagens (types I, II, III, V, and XI) are the most important molecules in conferring mechanical strength. They are synthesized and secreted as procollagens with large globular N- and C-terminal propeptides, which are proteolytically processed to generate mature collagen. These triple-helical molecules, composed of three α-chains with series of Gly-X-Y triplet sequences, are highly resistant to proteolysis. In tissues, individual 2 nm thick collagen molecules assemble into 20-200 nM diameter fibrils. Covalent crosslinking stabilizes these fibrils so that they associate laterally to form fibers. Type I collagen is the major component of collagen fibrils in a variety of tissues including bone, skin, tendon, and other fibrous tissues. It is a heterotrimer of two α1(I) and one α2(I) chains. Type II collagen, the main component of cartilage, is a homotrimer α1(II)3. Type III [α1(III)3] and V [α1(V), α2(V), α3(V)] collagens form heterotypic fibrils with type I collagen in soft connective tissues. In bone, type I collagen is accompanied by type V and XI [α1(XI), α2(IX), α3(XI)] collagens. Type XI collagen also forms heterotypic fibrils with type II collagen (for a review, see Ottani et al., 2002).

2) Non-fibrillar collagens form a rather heterogeneous group (Aumailley and Gayraud, 1998; Prockop and Kivirikko, 1995). Fibril-associated collagens with interrupted triple-helices (FACIT-collagens, type IX, XII, XIV, XVI, and XIX) are found in association with collagen fibrils. Type VI collagen forms microfibrils in most stromal connective tissues. The network-forming collagens are found in hypertrophic cartilage (type X), subendothelial matrices

(type VIII), and basement membranes (type IV). Long-chain collagen (type VII) is a component of anchoring fibrils, which stabilize the attachment of basement membranes and epithelia to underlying stroma. In addition, collagens with a transmembrane domain (type XIII and XVII), multiplexin (multiple triple-helix domain and interruptions) collagens (type XV and XVIII), and other proteins containing triple-helical domains have been characterized (Prockop and Kivirikko, 1995).

Table 1.1. Some types of collagen and their properties

(reproduced from Alberts B., 2002)

	Type	Molecular Formula	Polymerized Form	Tissue Distribution
Fibrillar	**I**	$[\alpha1(I)]_2\alpha2(I)$	fibril	Bone, skin, tendons, ligaments, cornea, internal organs
	II	$[\alpha1(II)]_3$	fibril	Cartilage, invertebral disc, notochord, vitreous humor of the eye
	III	$[\alpha1(III)]_3$	fibril	Skin, blood vessels, internal organs
	V	$[\alpha1(V)]_2\alpha2(V)$ and $\alpha1(V)\alpha2(V)\alpha3(V)$	fibril (with type I)	As for type I
	XI	$\alpha1(XI)\alpha2(IX)\alpha3(XI)$	fibril (with type II)	As for type II
Non-fibrillar	**IX**	$\alpha1(IX)\alpha2(IX)\alpha3(IX)$	lateral association with type II fibrils	Cartilage
	XII	$[\alpha1(XII)]_3$	lateral association with	Tendons, ligaments, some

			type I fibrils	other tissues
Network-forming	**IV**	$[\alpha1(IV)]_2\alpha2(IV)$	sheetlike network	Basal lamina
	VII	$[\alpha1(VII)]_3$	anchoring fibrils	Beneath stratified squamous epithelia
Transmembrane	**XVII**	$[\alpha1(XVII)]_3$	not known	Hemidesmosomes
Others	**XVIII**	$[\alpha1(XVIII)]_3$	not known	Basal lamina around blood vessels

Note that types I, IV, V, IX and XI are each composed of two or three types of α chains, whereas types II, III, VII, XII, XVII and XVIII are composed of only one type of α chain each. Only 11 types of collagen are shown, but about 20 types of collagen and about 25 types of α chains have been identified so far.

Elastic fibers confer elastic properties to tissues like lung, dermis, and large blood vessels (Rosenbloom et al., 1993). Elastin, the main component of elastic fibers, is a hydrophobic and extensively crosslinked protein, resistant to harsh physical treatments and to most proteinases. The elastic fibers interact with microfibrils consisting of fibrillins, other glycoproteins, and proteoglycans (Debelle and Tamburro, 1999; Saharinen et al., 1999).

Various **glycoproteins** are present in the interstitial ECMs. Fibronectin and vitronectin are structural ECM glycoproteins also present in plasma, which mediate cell attachment to the ECM (Tryggvason et al., 1987). Fibronectins are high-molecular mass (235-270 kDa) glycoproteins that form disulfide-linked dimers and fibrillar structures. Extensive alternative splicing of a single gene generates different forms of fibronectins. They are composed of three types of repeats, and other functional domains. Fibronectins interact with cell surface integrin receptors principally through an RGD-sequence. They also bind to different matrix components including collagens, fibrin, and proteoglycans. During development, adhesion of embryonic cells to fibronectin is essential for

their migration through fibronectin rich matrixes (George et al., 1993). Upon cell transformation, decreased synthesis and enhanced proteolytic degradation downregulates fibronectins at the cell surface and within the ECM (Tryggvason et al., 1987; Vartio et al., 1983b). Many other glycoproteins associate with various structural ECM elements. Chondronectin, for example, is a cell-associated glycoprotein in cartilage, which promotes the attachment of chondrocytes to type II collagen. Thrombospondins, tenascins, and SPARC (secreted protein, acidic and rich in cysteine) are capable of mediating both adhesive and anti-adhesive interactions and can induce disassembly of focal contact structures (Murphy-Ullrich, 2001).

Proteoglycans (PGs) and glycosaminoglycans (GAGs) constitute the amorphous substance of interstitial ECM, a highly hydrated, gel-like "ground substance" in which the fibrous proteins are embedded. The polysaccharide gel resists compressive forces on the matrix while permitting the rapid diffusion of nutrients, metabolites, and hormones between the blood and the tissue cells. The collagen fibers both strengthen and help organize the matrix, and elastin fibers give it resilience.

PGs consist of a core polypeptide chain, in which serine and threonine residues have O-linked GAG chains of heparan, keratan, dermatan, or chondroitin sulfate (Iozzo, 1998). The turnover of PGs is faster than that of collagens, with half-lives in tissues of a few days to several weeks. PGs not only form structural frameworks and act on matrix organization, but also play a role in the modulation of variety of biological processes including cell growth, adhesion, and invasion (Schwartz, 2000). Many ECM-associated growth factors, such as FGF and VEGF family members, bind to heparan sulphate PGs (Taipale and Keski-Oja, 1997). Small, leucine-rich PGs, such as decorin and fibromodulin, bind to ECM components and participate in the regulation of collagen fibrillogenesis and organization of the matrix (Iozzo, 1999). They also bind TGF-β and may thus modulate the biological effects of this growth factor (Yamaguchi et al., 1990).

Aggregan and versican, the main chondroitin sulphate PGs of cartilage and noncartilagenous tissues, respectively, interact with glycoproteins and hyaluronic acid to create extensive networks (Wight et al., 1992). Cell surface-associated PGs such as glypicans, syndecans, and CD44 modulate cell adhesion. Recent evidence indicates the importance of cleaving some of these PGs (e.g. syndecan-1, CD44) in promoting tumor invasion and metastasis (reviewed in Inki and Jalkanen, 1996; Nagano and Saya, 2004).

1.1.2 Basement membrane

The term *basement membrane* is often used to describe the composite of the basal lamina and the layer of anchoring collagen fibrils tethering the basal lamina to the underlying connective tissue. Basal laminae (BL) are specialized extracellular matrix sheets that separate epithelial and endothelial cell layers from the underlying cells of the collagenous stroma (Timpl, 1996). BL have more than simple structural roles, however. They are able to determine cell polarity, influence cell metabolism, organize the proteins in adjacent plasma membranes, promote cell survival, proliferation, or differentiation, and serve as specific highways for cell migration (Alberts *et al.*, 2002). They are produced and assembled in co-operation by cells situated on both sides of the basement membrane.

The main BL components include type IV collagen, laminin, entactin/nidogen, and heparan and chondroitin sulfate proteoglycans (PGs). PGs are present in all BL structures where they may function in charge-dependent molecular sieving and the immobilization of growth factors like FGF-2 and VEGF, which can bind to perlecan, the main BL heparan sulfate PG (Handler et al., 1997; Iozzo, 2001).

Type IV collagens are trimeric proteins (~540 kDa) composed of three parallel α(IV) chains, which form a partially triple-helical structure with numerous interruptions (Timpl, 1996). Six different chains have been cloned

(α1(IV) to α6(IV)). The α1(IV) and α2(IV) chains are found in most basement membranes, whereas the expression of α3-6(IV) chains is more restricted (Hudson et al., 1993; Yurchenco and O'Rear, 1994b). The most common trimer is $\alpha1(IV)_2\alpha2(IV)$. Type IV collagen trimers can assemble into a three-dimensional network through three types of interactions (Timpl, 1996; Yurchenco and O'Rear, 1994b). Four molecules can bind to each other at the N-terminal cysteine-rich domain (7S) to form tetramers, which are joined to a network structure by a dimeric interaction between C-terminal non-collagenous (NC1) domains. Additional lateral associations of collagen molecules lead to the formation of irregular three-dimensional networks, which are stabilized by disulfide bonds and covalent cross-links (see also Table 1.1).

Laminins are a group of large heterotrimeric glycoproteins (~400-900 kDa) consisting of three distinct polypeptide chains: α, β,and γ chains (Tryggvason, 1993). At least twelve different heterotrimeric laminin isoforms, assembled from five α, three β, and three γ chains, have been characterized with different tissue distributions and functions (Colognato and Yurchenco, 2000; Tryggvason, 1993; Yurchenco and Wadsworth, 2004). Like type IV collagen, laminins also self-assemble through calcium-dependent interactions involving the terminal domain of three chains to form a polymer network *in vitro* (Timpl, 1996; Yurchenco and O'Rear, 1994b).

The networks of type IV collagen and laminin are connected through various interactions. For example, nidogen is a sulfated glycoprotein (150 kDa), which bridges these molecules together (Colognato and Yurchenco, 2000). It has a C terminal binding site for the EGF repeat of laminin γ chains that is located near the center of laminin trimer, and an N-terminal binding site for type IV collagen. In addition, perlecan contains binding sites for both type IV collagen and laminin (Kallunki and Tryggvason, 1992). The NC1 domains of type XV and XVIII collagens also bind to perlecan and laminin/nidogen complexes (Sasaki et

al., 1998b). Much of the information required for the assembly of complicated ECM structures including collagen fibrils and type IV collagen and laminin networks appears to be intrinsic to the molecules. Indeed, these components can be induced to self-assemble *in vitro* in cell-free systems to structures resembling those found *in vivo* (Yurchenco and O'Rear, 1994a). In contrast, fibronectin fibrillogenesis is controlled by cell surface integrins and cytoskeletal components (Magnusson and Mosher, 1998), and in tissues, basement membrane assembly may also be regulated through cell-cell interactions.

1.2 Cross-talk between the extracellular matrix and cells

A dynamic reciprocal interplay between cells and the ECM environment is the basis of a flow of information that regulates cell survival, proliferation, differentiation, and migration during different biological processes (i.e. embryonic development) (Zagris et al., 2004). The major players mediating and modulating this information not only include ECM components, growth factors, cytokines, integrins and intracellular signal transduction cascades but also extracellular matrix modifying enzymes (Streuli, 1999) (Fig. 1.2).

In normal adult tissues, ECM remodeling and cell proliferation are slow, the capacity for cell motility is generally repressed, and stable cell adhesion is essential for the maintenance and function of the tissues. For example, epithelial cells receive signals for quiescence through interactions with intact basement membrane and neighbouring cells (Radisky et al., 2001). In the absence of specific ECM interactions, cells fail to respond correctly to growth factors and maintain or achieve an appropriate phenotype (Streuli, 1999). Consequently, the cells function inappropriately or die. Indeed, changes in the ECM, adhesive interactions, and ECM remodeling machinery are the first to take place during malignant transformation and metastasis (Radisky and Bissell, 2004; Yap, 1998).

ECM can affect cell behavior by storing and mobilizing growth factors and cytokines.

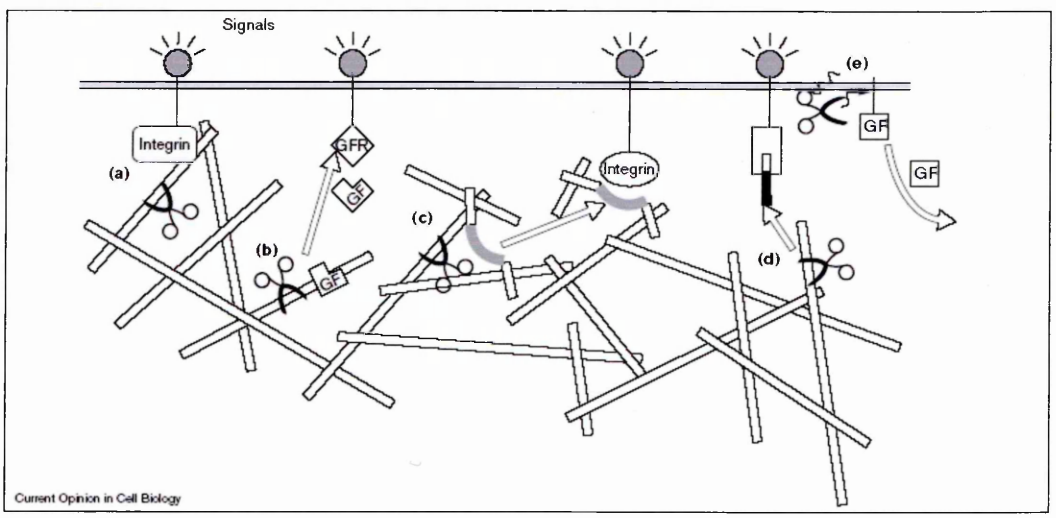


Fig. 1.2. Some ways in which ECM remodeling can affect cellular differentiation (reproduced from Streuli C., 1999). (a) Remodelled ECM directly alters cell–ECM interactions, affecting integrin signalling and integrin-mediated crosstalk with growth factors. **(b)** ECM remodelling releases bound growth factors (GF). **(c)** Exposure of cryptic sites (grey bands) within the ECM alters integrin signalling. **(d)** ECM remodelling releases bioactive ECM fragments (black bar). **(e)** Surface-bound ECM remodelling enzymes shed growth factor ectodomains. GFR, growth factor receptor.

These signal to cells through receptor tyrosine (or serine/threonine) kinases and receptors coupled with either G-proteins or signaling complexes consisting of tyrosine or serine/threonine kinases and phosphatases. The remodeling of ECM components, and the release or activation of ECM-bound growth factors by proteolysis, both modulate transduced signals, which in turn control cell phenotype. Some of these changes participate in the modulation of proteolysis by feedback signaling (Taipale and Keski-Oja, 1997). Interestingly, a distinct subfamily of discoidin domain tyrosine kinase receptors (DDR1 and DDR2), which signal in response to collagens rather than growth factors, have also been identified (Alves et al., 1995; Vogel et al., 1997). These receptors can also establish a feedback loop between cells and pericellular proteolysis (Olaso et al., 2002).

Cells attach and react to the surrounding ECM also through a large diversity of adhesion receptors including integrins, heterodimeric transmembrane glycoproteins composed of noncovalently associated α- and β-chains. The combination of α- and β-chains provides each integrin with a unique range of specificities for different ECM components and cell surface counter receptors (Ruoslahti and Obrink, 1996). Clustering of integrins by ligand-specific interactions induces cytoskeletal accumulation of multiprotein complexes composed of integrins associated with interacting adaptor proteins and signaling molecules. Intracellular events can then feed back on the expression and activity of the integrins and other gene products of the cell. The response that a particular cell makes depends on the composition and architecture of the ECM network as well as on the repertoire of cell receptors expressed.

1.3 Cell migration: an interplay between extracellular matrix and pericellular proteolysis?

Binding of ECM components to specific cell surface integrins not only

modulates the integrin activity but also induces integrin clustering into focal contacts at the leading edges of migrating cells. The cytoplasmic tails of integrins interact with cytoskeletal multiprotein complexes that link the focal contacts to actin filaments and intracellular signaling pathways. These interactions regulate the migratory potential of cells.

Cell migration is a recurring phenomenon in all morphogenic processes during embryonic development (Zagris, 2001). In adults, physiological migration processes include trophoblast invasion and invasive migration by neutrophils and macrophages (Mignatti and Rifkin, 2000). In many other adult cells the capacity for cell movement can be activated by wounding or trauma or by malignant transformation. Cell migration is accomplished by propulsive forces transferred from the ECM to cytoskeleton through repeated cycles of adhesion, detachment and proteolysis. These cycles are regulated by the activity of Rho family of small GTPases such as Cdc42, Rac and Rho, which are critical for the assembly of actin cytoskeletal components to form membrane ruffles, filopodia, and lamellipodia (Lauffenburger and Horwitz, 1996; Mitchison and Cramer, 1996).

The direction of cell migration is mainly affected by chemoattractants and by the generation of preferred adhesive interactions. Chemoattractants include cytokines and growth factors such as TGF-α, EGF, FGF, and VEGF as well as ECM fragments, which are recognized by adhesion receptors.

Proteolytic enzymes and their inhibitors may thus modulate cell migration by: 1) clearing the restricting ECM, 2) remodeling ECM components or cell adhesion molecules to modify the affinity and dynamics of cell-ECM interaction, 3) mobilizing and activating or inactivating growth factors, cytokines, and chemokines (Fowlkes et al., 1994; Imai et al., 1997; McQuibban et al., 2001; Whitelock et al., 1996), 4) generating bioactive peptides such as endostatin (Sasaki et al., 1998a; Wickstrom and Carlstedt, 2001), angiostatin (Cornelius et al., 1998), and sites in laminin-5 α3 chain that may direct the migration front of invading cells (Giannelli et al., 1997; Koshikawa et al., 2000).

1.4 Extracellular matrix remodeling

Coordinated degradation of ECM is involved in various physiological tissue remodeling processes such as tissue morphogenesis and growth, angiogenesis, trophoblast implantation, bone remodeling, wound healing, and involution of postpartum uterus or postlactation mammary gland (Mott and Werb, 2004; Sternlicht and Werb, 2001; Vu and Werb, 2000; Zagris, 2001). Both excessive and deficient proteolysis are associated with a number of pathological conditions like arthritis, periodontitis, chronic wounds, and scleroderma (Birkedal-Hansen, 1995; Sternlicht and Werb, 2001; Mott and Werb, 2004). During tumor invasion, neoplastic cells utilize proteolytic and invasive mechanisms in a controlled but abnormally regulated fashion to allow cell attachment, localized degradation of the ECM, and cell migration through the digested barrier (Stetler-Stevenson and Yu, 2001). Remodeling of the differentiated ECMs of various organs and the subepithelial or subendothelial basement membranes is dependent on the focal and concerted action of several proteinases from different proteinase families.

Focal proteolytic activity can be achieved when proteinases are compartmentalized and activated in the immediate pericellular environment where proteinase inhibitors, derived either from plasma or secreted by the tissue cells, have limited access (Sternlicht and Werb, 2001). Indeed, the localization of proteases and their activators to the plasma membrane and special membrane domains via a transmembrane domain or binding to cell surface receptors has emerged as an important mechanism for the generation of proteolytic activity which then mediates a variety of cellular functions (Sternlicht and Werb, 2001).

The cell surface enzymes characterized in pericellular proteolysis include matrix metalloproteinases (MMPs), adamalysin-related metalloproteinases (ADAMs), and serine proteases including type II transmembrane serine proteinases (TTSPs), plasmin and the urokinase plasminogen activator (uPA) / uPA receptor system (Blobel, 1997; Hooper et al., 2001). All these proteinases

have multidomain structures that provide them with capacity to interact with multiple partners in many cases on both sides of plasma membrane and in intracellular compartments. Therefore, they may be involved in multiple cellular pathways.

During cell invasive and morphogenic processes, cell surface association of proteolytic activity has several advantages. First, the proteinase-inhibitor balance can be finely tuned to favor pericellular proteolysis, while the surrounding tissue is protected by inhibitors. As a result, sufficient matrix can be degraded to allow forward movement while an attachment surface is left for propulsion. Secondly, cell surface-associated proteinases may specifically interact with cell surface signaling receptors or their pericellular ligands, as well as with the cytoplasmic signaling complexes, and in this manner function as key regulators of signaling events. Thirdly, proteolytic enzymes may interact and cooperate with adhesion receptors to control the continuous cell adhesion and detachment events required for cell movement (Werb, 1997; Hiraoka et al., 1998; Hotary et al., 2000).

For example, the ADAMs that are thought to be involved in fertilization, development, inflammation, and angiogenesis (Black et al., 1997; Blobel, 1997) have been implicated in the proteolysis of ECM components and cell surface proteins (including Notch and TNF-α), in RGD-dependent and -independent interactions with integrins, and in signaling via interactions of their cytoplasmic domains (Black et al., 1997).

The uPA/uPA receptor (uPAR) system, on the other hand, is an example of complex coordinated regulation of protease and adhesion systems to promote cell migration. While uPAR is the major binding site for uPA, the plasminogen activator generating active plasmin (Chapman, 1997), uPAR may also serve as a high affinity receptor for vitronectin (Wei et al., 1994). Based on the observation that macrophage adhesion is affected by uPA binding to uPAR independently of the proteolytic activity of uPA, uPAR has been suggested to transduce signals to

cytoskeleton that regulate cell adhesion (Gyetko et al., 1994).

1.5 Matrix metalloproteinases

Proteases are classified into exo- or endopeptidases according to the terminal or internal cleavage site on the target proteins respectively (Woessner, 1998). Endopeptidases are divided into the major classes of serine, cysteine, aspartic, and metalloproteinases based on their amino acid sequences and the cofactors determining their catalytic activity and mechanism. Matrix metalloproteinases (MMPs) form one of the four subfamilies that belong to metzincins, which in turn is one of numerous metalloproteinase superfamilies (Sternlicht and Werb, 2001; Woessner, 1998).

MMPs have historically been categorized in four subgroups on the basis of their specificity for ECM components: collagenases, gelatinases, stromelysins and matrilysins (Sternlicht and Werb, 2001). As the list of MMPs is growing, a numbering system has been adopted and MMPs are now classified according to their structure. Eight structural classes are recognized, five of which are secreted MMPs, and three membrane-type MMPs (MT-MMPs) (reviewed in Mook et al., 2004).

The MMP family currently consists of 28 distinct but structurally related vertebrate enzymes, with 24 characterized human homologues that have partially overlapping substrate specificities (Freije et al., 2003; Overall and Lopez-Otin, 2002; Sternlicht and Werb, 2001) (Fig. 1.3). They are all zinc-dependent neutral endopeptidases, whose activation requires the removal of the N-terminal prodomain. MMPs function in the degradation and remodeling of different ECM proteins and proteoglycans, but recent studies also suggest various other roles for these enzymes. For example, MMPs can cleave membrane or ECM-bound cytokine precursors, chemokines, growth factors, hormone receptors, growth factor binding proteins, proteinase inhibitors, and thus modulate their assembly and activity (for a review, see Sato et al., 2005).

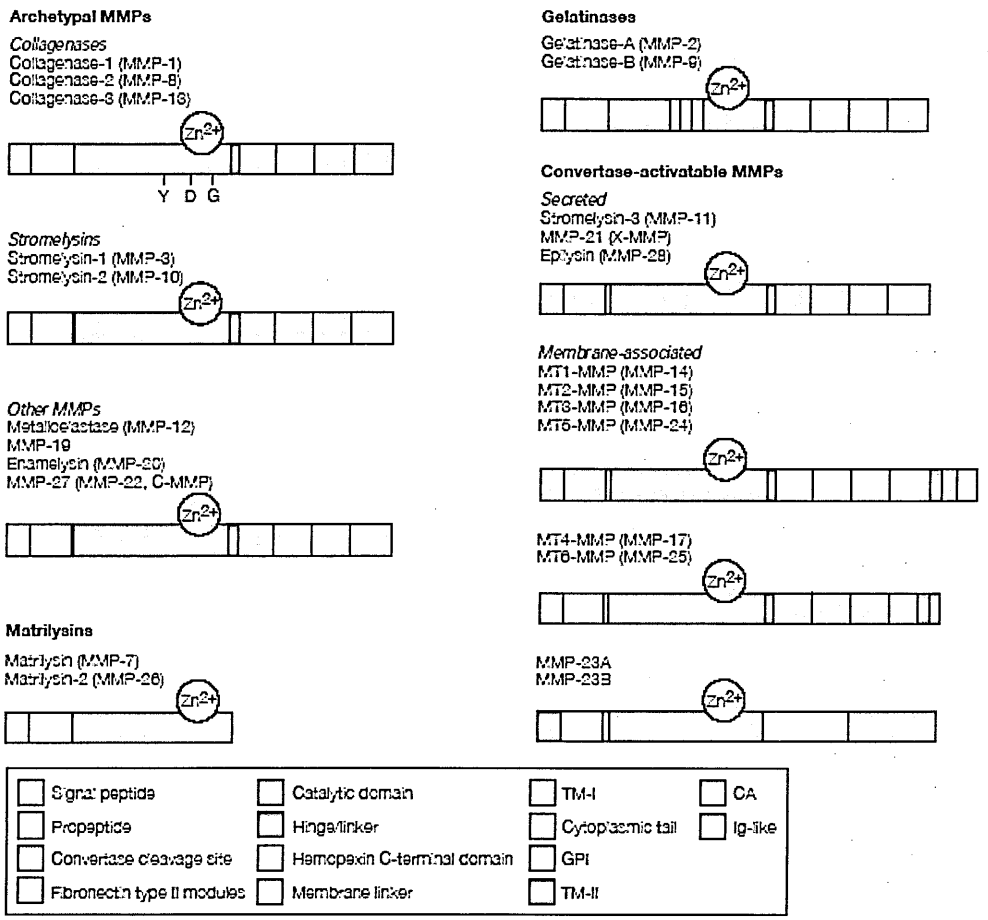


Fig. 1.3. Schematic representation of the structure of the 24 human matrix metalloproteinases (MMPs), classified into different groups on the basis of domain organization (reproduced from Overall and Lopez-Otin, 2002). Archetypal MMPs contain a signal peptide (necessary for secretion), propeptide, a catalytic domain that binds zinc (Zn2+) and a hemopexin carboxy (C)-terminal domain. Y, D, and G represent tyrosine, aspartic acid and glycine amino acids that are present in the catalytic domain of all collagenases. Matrilysins contain the minimal domain organization that is required for secretion, latency and catalytic activity. Gelatinases contain fibronectin type II modules that improve collagen and gelatin degradation efficiency. Convertase-activatable MMPs contain a basic insert in the propeptide that is targeted by furin-like proteases (convertase cleavage site). MMPs that belong to this group can be secreted enzymes, or membrane-anchored via GPI (glycosylphosphatidylinositol), type I or type II transmembrane (TM) segments. MMP-23A and MMP-23B contain unique cysteine array (CA) and immunoglobulin (Ig)-like domains in their C-terminal region.

MMP activity is regulated by diverse mechanisms at the levels of gene transcription, mRNA stability, enzyme secretion and binding, zymogen activation, and inhibition by endogenous inhibitors to achieve precise proteolysis during normal tissue remodeling (Nagase and Woessner, 1999). Dysregulated MMP activity is typical of a number of pathological conditions such as chronic wounds, arthritis, periodontitis, cardiovascular disease, and cancer.

The vast majority of MMPs contain characteristic protein domains including from the N-terminal to the C-terminal extremities: a signal peptide, a propeptide, a catalytic domain with the zinc binding site, a hinge region and a C-terminal hemopexin-like domain - hemopexin is a serum glycoprotein that binds heme groups reversibly and delivers them to the liver where they are taken up by receptor-mediated endocytosis - that is thought to confer some degree of substrate specificity (Sternlicht and Werb, 2001).

MMPs can be divided into subgroups based on structural and functional criteria. They are either soluble proteins or membrane proteins anchored to cellular membranes by type I or type II transmembrane domains or by a glycosyl-phosphatidyl inositol (GPI)-anchor. The soluble secreted MMPs have been further classified in the subgroups of **collagenases**, which can degrade fibrillar collagens, **gelatinases**, with high activity against gelatin and type IV collagen, and **stromelysins, matrilysins** and **other MMPs**, which degrade a variety of ECM components (see Table 1.2). The membrane-anchored MMPs form the group of the so-called **membrane-type matrix metalloproteinases** and they will be described in-depth in section 1.6.

Table 1.2. Secretory matrix metalloproteinases

Enzyme name(s)	*ECM substrates*	*Other substrates*
Collagenases		
MMP-1/Collagenase-1	Col I, II, III, VII, VIII, X,	ProMMP-1, 2; casein, α2M; α1-PI;

	XI; gelatin; entactin; aggrecan; tenascin; MBP; perlecan; IGF-BP-2, 3	α2AC; proTNFα
MMP-8/Collagenase-2	Col I, II, III, gelatin; entactin; aggrecan; tenascin	ProMMP-8; α2M; α1-PI
MMP-13/Collagenase-3	Col I, II, III, IV, IX, X, XIV; gelatin; entactin; aggrecan; tenascin; osteonectin; fibrinogen/fibrin	ProMMP-9; 13; α2M; α2AC; PAI

Gelatinases (type IV collagenases)

MMP-2/Gelatinase A	Gelatin; elastin; fibronectin; Col I, IV, V, VII, X, XI; laminin; aggrecan; vitronectin; decorin; MBP; IGF-BP-3/5	ProMMP-1, 2, 13; plasminogen; casein; α2M; α1-PI; α2AC; proTNFα; proTGFβ2; proIL1β; MCP3; FGFr1
MMP-9/Gelatinase B	Gelatins; Col IV, V, VII, XI, XIV, XVII; elastin; fibrillin; fibronectin; aggrecan; fibrinogen/fibrin; MBP	Plasminogen; casein; α2M; α1-PI; proTNFα; proTGFβ2; proIL1β

Stromelysins, matrilysins and others

MMP-3/Stromelysin-1	Fibronectin; laminin; gelatin; Col III, IV, V, VII, IX, X, XI; elastin; decorin; nidogen; perlecan; aggrecan; tenascin; fibrin/fibrinogen; fibrillin; entactin; vitronectin; IGFBP-3	ProMMP-1, 3, 7, 8, 9, 13; plasminogen; casein; α2M; α1-PI; α2AC; proTNFα; E-cadherin; proIL-1β; proHB-EGF
MMP-10/Stromelysin-2	Fibronectin; laminin; gelatin; Col III, IV, V, VII, IX, X, XI; decorin; elastin; nidogen; fibrin/fibrinogen; fibrillin; entactin; tenascin; vitronectin; aggrecan	ProMMP-1, 8, 10
MMP-11/Stromelysin-3	Laminin; fibronectin; aggrecan; IGF-BP-1	α2M; α1-PI
MMP-7/Matrilysin-1	Fibronectin; laminin; Col IV; gelatin; aggrecan; decorin; nidogen; elastin; fibrillin; laminin; MBP; osteonectin; tenascin; vitronectin	ProMMP-2, 7; casein; α1-PI; pro α defensin; FasL; β4 integrin; E-cadherin; plasminogen; proTNFα
MMP-26/Matrilysin-2	Col IV; gelatin; fibronectin; fibrin/fibrinogen	ProMMP-9; casein; α1-PI
MMP-12/Macrophage metalloelastase	Elastin; fibronectin;	Plasminogen; casein

	fibrinogen/fibrin; laminin	
MMP-19	Col IV; gelatin; fibronectin; tenascin; aggrecan; COMP	
MMP-20/Enamelysin	Amelogenin; aggrecan; COMP	
MMP-28/Epilysin	ND	Casein

Modified from (Mc Cawley and Matrisian, 2001: Sternlicht and Werb, 2001). Abbreviations: Col, collagen; COMP, cartilage oligomeric matrix protein; IGF-BP, insulin-like growth factor binding protein; Ln, laminin; MBP, myelin basic protein; PAI, plasminogen activator inhibitor; α2M, α2 macroglobulin;α1-PI, α1 proteinase inhibitor; α2AC, α2 antichymotrypsin; ND, not determined.

1.5.1 Collagenases

Collagenases-1, -2, and -3 (now referred to MMP-1, MMP-8, and MMP-13, respectively) are the main secreted neutral proteinases, which can initiate the degradation of native helix of fibrillar collagens (Reynolds, 1996). The hemopexin domains of these MMPs are essential for specific binding and cleavage of this substrate (Allan et al., 1995; Clark and Cawston, 1989). All three collagenases can cleave a specific site (Gly775-Ile/Leu776) at each α-chain of the trimeric collagen molecule. The resulting N-terminal 3/4 and C-terminal 1/4 fragments are spontaneously denatured at 37°C to gelatin, which can be further degraded by other proteinases (Ala-aho and Kahari, 2005) (Fig. 1.4). Three collagenases have overlapping activities on the fibrillar collagen types I, II, and III, but their substrate preferences differ (see Table 1.2).

MMP-1 was the first MMP to be discovered based on its activity in the metamorphosing tadpole tail (Gross and Lapiere, 1962). It was also the first MMP purified to homogeneity (Stricklin et al., 1977) and cloned as a cDNA (Goldberg et al., 1986). MMP-1 is secreted *in vitro* by fibroblasts of various origins, chondrocytes, osteoblasts, endothelial cells, keratinocytes, hepatocytes, macrophages and monocytes, eosinophils and tumor cells.

MMP-8 is synthesized by polymorphonuclear leukocytes during their

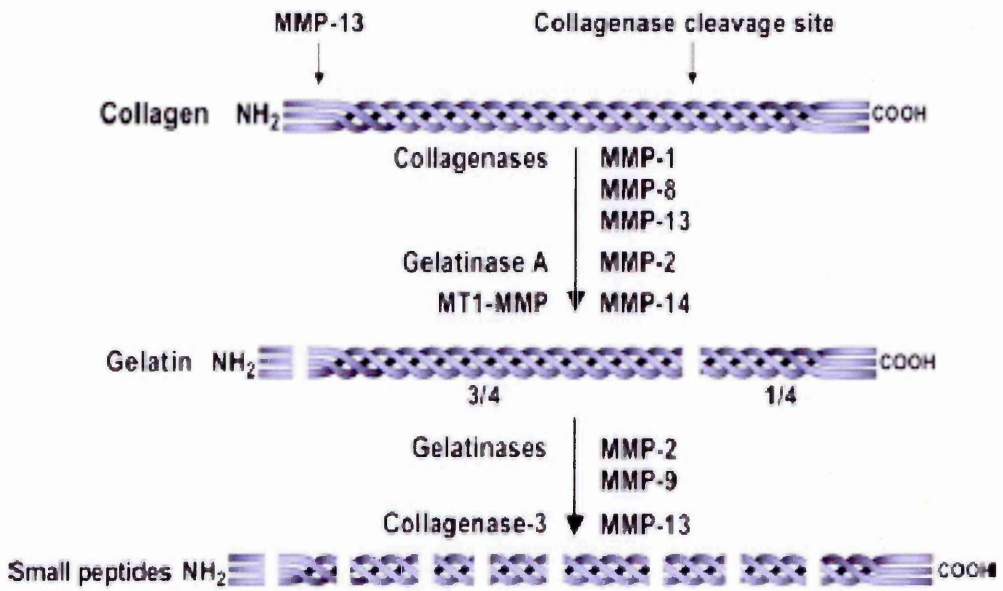


Fig. 1.4. Degradation of fibrillar collagens (reproduced by Ala-aho R. et al., 2005). Fibrillar collagens are degraded by collagenases, MMP-1, MMP-8 and MMP-13 at the specific site of the α chain resulting in generation of 3/4 N-terminal and 1/4 C-terminal fragments that are denaturated into gelatin at body temperature. Gelatin fragments are further degraded by gelatinases MMP-2 and MMP-9 but also by MMP-13. Furthermore MMP-13 cleaves type I collagen in non-helical telopeptide. Membrane bound MT1-MMP (MMP-14) can also cleave fibrillar collagen and MMP-2 shows weak collagenolytic activity.

maturation in bone marrow, stored in intracellular granules, and released in response to external stimuli (Hasty et al., 1990). It is also expressed by chondrocytes, rheumatoid synovial fibroblasts, gingival fibroblasts, bronchial epithelial cells and melanoma cells (Cole et al., 1996; Vernooy et al., 2004).

MMP-13 is expressed during fetal bone development, postnatal bone remodeling, and gingival wound repair (Ravanti et al., 1999). In addition, MMP-13 expression has been associated with pathological conditions such as severe chronic inflammation in osteoarthritic cartilage, rheumatoid synovium, and chronic wounds, as well as malignant tumor invasion (Airola et al., 1997; Balbin et al., 1999; Johansson et al., 1997; Vaalamo et al., 1997).

1.5.2 Gelatinases

MMP-2 (gelatinase A, 72-kDa gelatinase) and MMP-9 (gelatinase B, 92-kDa gelatinase) differ from the other MMPs in that they contain three head-to-tail repeats homologous to the type II repeat of the collagen-binding domain of fibronectin (Collier et al., 1988). These domains are required to bind and cleave collagen (Keski-Oja and Todaro, 1980; Keski-Oja and Vaheri, 1982; Murphy et al., 1994; Steffensen et al., 1995; Vartio, 1982; Vartio and Vaheri, 1981) and elastin (Shipley et al., 1996). The hemopexin domain does not affect MMP-2 binding to collagen (Allan et al., 1995), but similarly to collagenases, it is critical for the initial cleavage of the triple helical type I collagen (Patterson et al., 2001). The hinge domain of MMP-9 contains an additional type V collagen-like insert (Wilhelm et al., 1989).

A wide range of normal and transformed cells of fibroblastic, endothelial, and epithelial origin constitutively express MMP-2 (Collier, 2001; Huhtala et al., 1991; Salo et al., 1982; Tryggvason et al., 1990; Vartio and Vaheri, 1981). During development it is widely expressed by stromal cells (Reponen et al., 1992). Expression of MMP-9 is more restricted and is often low in normal tissues, but it can be induced when tissue remodeling occurs during development, wound

healing and cancer invasion. MMP-9 is secreted by alveolar macrophages, polymorphonuclear leukocytes, osteoclasts, keratinocytes, and invading trophoblasts, and by several transformed cell lines, but not by fibroblastic cells (Hibbs, 1992; Reponen et al., 1995; Saarialho-Kere et al., 1993; Vartio et al., 1983a).

Both gelatinases can degrade a variety of proteins *in vitro*, but the *in vivo* substrates are largely unknown (Sternlicht and Werb, 2001). Based on their ability to degrade type IV collagen and laminin (the main components of BM), the correlation between the levels of MMP-2 expression and activity, coupled with the invasive potential of certain cancer cells, gelatinases, especially MMP-2, have been suggested to degrade BM components *in vivo* (Tryggvason et al., 1987). Both gelatinases can also efficiently degrade partially denatured collagens of all types following the initial cleavage by collagenases (Overall et al., 1989). MMP-2, but not MMP-9, selectively cleaves the α2-chain of laminin 5 and promotes cell migration in a mammary epithelial cell model (Giannelli et al., 1997). MMP-9 has been suggested to affect angiogenesis by releasing ECM-bound VEGF (Vu et al., 1998).

1.5.3 Stromelysins, matrilysins and other MMPs

Stromelysins include stromelysin-1, -2 and -3 (MMP-3, MMP-10, and MMP-11, respectively). The domain structures of stromelysins resemble those of collagenases. However, they are unable to cleave native fibrillar collagens. Stromelysin-3 is inactive against many ECM components; instead it can cleave proteinase inhibitors, α2-macroglobulin (α2M) and α1-PI (Pei et al., 1994), and insulin-like growth factor binding protein (IGF-BP) (Manes et al., 1997). It also differs from most secreted MMPs by having recognition sequence for proprotein convertases between the pro- and catalytic domains. This would suggest a possible mechanism of post-translational regulation in its activity. Some recently characterized soluble MMPs including MMP-21 and epilysin (MMP-28) also

contain insertions with similar basic sequences (Pei, 1999; Velasco et al., 1999).

Matrilysins-1 and -2 (MMP-7 and MMP-26, respectively), macrophage metalloelastase (MMP-12), MMP-19 and enamelysin (MMP-20) are other MMPs with broad substrate specificities (see Table 1.2).

1.6 Membrane-type matrix metalloproteinases

The existence of membrane-bound MMPs was first suggested by the finding that plasma membranes from various tumor cells contained a proMMP-2 activator sensitive to MMP inhibitors (Brown et al., 1993; Strongin et al., 1993). Eventually, Sato *et al.* (Sato et al., 1994) cloned the cDNA for the first MT-MMP (MMP-14), encoding a 63-kDa type I transmembrane protein that could activate proMMP-2 and promote cell invasion. To date, additional cDNAs for membrane-bound MMPs have been cloned. Their six protein products have been named MT-MMP-2, -3, -4, -5, and -6, and Cysteine Array-MMP (MMP-15, -16, -17, -24, -25, and -23, respectively) (Zucker et al., 2003).

MT-MMPs have recently gained considerable attention, basically because of the marked abnormalities associated with MT1-MMP knockout mice in contrast to the more subtle phenotypes of mice deficient in secretory MMPs (Holmbeck et al., 1999; Shapiro, 1998; Sounni and Noel, 2005; Zhou et al., 2000). MT1-MMP is also more active in ECM degradation and promoting cell invasiveness in experimental models than its soluble form or the secretory MMPs, highlighting the importance of cell surface localization and cellular regulation of these enzymes (Chun et al., 2004; Hiraoka et al., 1998; Holmbeck et al., 1999; Hotary et al., 2000; Hotary et al., 2003; Sabeh et al., 2004). For this reason, I will now describe the general structural and functional features of MT-MMPs, and I will then focus my introduction on MT1-MMP (see section 1.8), not only for its biological relevance but also because it is the main topic of my experimental work.

1.6.1 Structure and activation of MT-MMPs

The MT-MMPs contain the protein domains characteristic of MMPs including from the N-terminal to the C-terminal extremities; a signal peptide, a propeptide, a catalytic domain with the zinc binding site, a hinge region and a C-terminal hemopexin domain (Figures 1.5 and 1.6). MMP-23 differs from all others, however, by having unique cysteine-rich, proline-rich, and IL-1 type II receptor-like domains instead of the C-terminal hemopexin-like domain (Pei, 1999) It also has an atypical N-terminal prodomain that lacks the conserved "cysteine switch" sequence, but contains a potential membrane spanning region. This binds proMMP-23 to the cellular membranes as a type II transmembrane protein. A single proteolytic cleavage between the pro- and catalytic domains by furin both activates the proenzyme and releases the activated soluble enzyme from the cell membrane (Pei et al., 2000).

In any case, all MT-MMPs are produced as zymogens containing a secretory signal sequence and a propeptide whose proteolytic cleavage is required for MMP activation. The prodomain of MT-MMPs contains a conserved motif, Tyr42-Gly43-Tyr44-Leu45, that acts as an intramolecular chaperone. It appears essential for adequate protease folding and enzymatic activities, including proMMP-2 activation, substrate degradation and tissue inhibitor of metalloproteinases-2 (TIMP-2) binding (Pavlaki et al., 2002). A basic tetrapeptidic sequence Arg108–Arg109–Lys110–Arg111 (RRKR) is inserted between the propeptide and the catalytic domain. This furin recognition motif is cleaved by proprotein convertases during the trafficking of MT-MMPs from the endoplasmic reticulum to the plasma membrane, although the precise localization of this event is still unclear (Cao et al., 1998; Kang et al., 2002; Mayer et al., 2003; Yana and Weiss, 2000). However, the activation of MT-MMPs by a furin-independent alternative pathway has been reported in some types of cells (Cao et al., 1996; Rozanov et al., 2001; Sato et al., 1996). This alternative activation pathway depends on an autoproteolytic activation or on the action of non-furin

Fig. 1.5. Comparison of the amino acid sequences of human MT-MMPs (reproduced from Velasco et al., 2000). The amino acid sequences of human MT-MMPs were extracted from the SwissProt database, and the multiple alignment was performed with the PILEUP program of the GCG package. Conserved residues in all human MT-MMPs are *shaded*. MT6-MMP sequence is shown in *bold*.

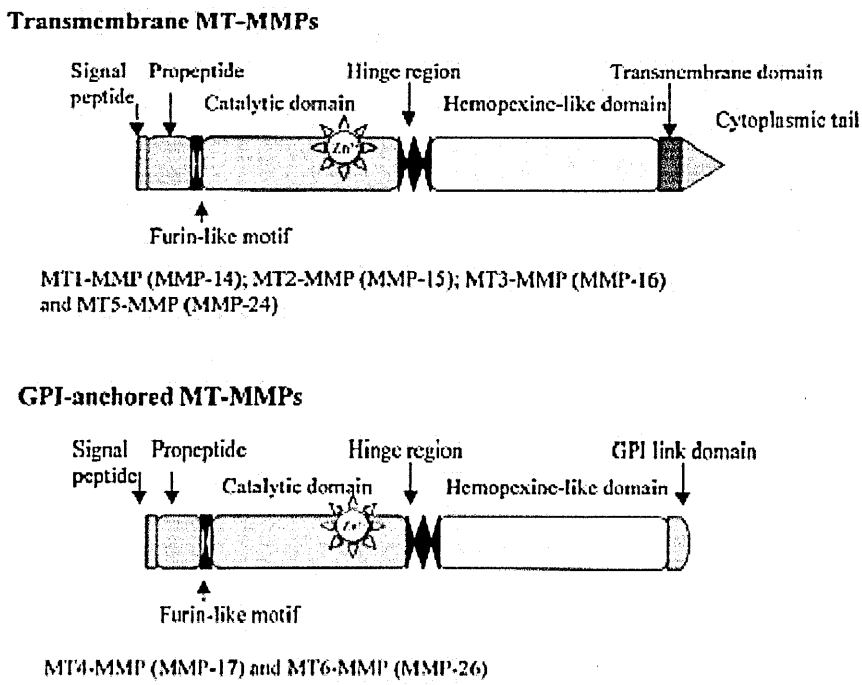


Fig. 1.6. The protein structure of MT-MMPs (reproduced from Sounni et al., 2005). Human membrane type matrix metalloproteinases (MT-MMPs) contain the basic common domains of almost MMPs: signal, pro-, catalytic, and hinge domains. MT1-, MT2-, MT3-, and MT5-MMP belong to the transmembrane-anchored type and are characterized by a transmembrane and a cytoplasmic domain. MT4-MMP and MT6-MMP are anchored to the plasma membrane via glycosylphosphatidylinositol, and belong to the GPI group.

proprotein convertases, or on other proteases located at the plasma membrane. In addition, an extracellular activation of proMT1-MMP by plasmin has been reported (Okumura et al., 1997). This debated aspect will be described in-depth in section 1.8.3.2 regarding the activation of MT1-MMP because its characterization is one of the main topics of my thesis.

The propeptide is followed by the catalytic domain containing the zinc-binding consensus motif HEBXHXBGBXH, where X is a variable residue and B is a bulky hydrophobic amino acid. The catalytic domain of MT-MMPs contains a characteristic 8-amino acid insertion between strands βII and βIII named the "MT-loop" (English et al., 2001a). The 3D structure of the complex between the catalytic domain of MT-MMPs and TIMP-2 shows that the MT-loop generates a pocket in the MMP catalytic domain fold that interacts with TIMP-2 (Lang et al., 2004). Although mutations in or deletions of the MT-loop of MT-MMPs do not affect its catalytic activity towards synthetic substrates, it impairs the kinetic of proMMP-2 activation (English et al., 2001a), whose mechanism will be described in section 1.6.3.1. Consistently, divergences in MT-loop structure between MT-MMPs are translated into their ability to activate proMMP-2. For example, MT4-MMP and MT6-MMP which lack the MT-loop are either unable to activate or inefficient at activating proMMP-2 (English et al., 2001a). Furthermore, human MT2-MMP is somewhat defective in cell-mediated activation of proMMP-2, whereas mouse MT2-MMP is very efficient in this activity. Indeed, the replacement of two residues (Pro183 and Glu185) in the MT-loop of the human enzyme by the corresponding ones (Ser183 and Asp185) of mouse MT2-MMP endowed the human enzyme with efficient activation of proMMP-2 (Miyamori et al., 2000).

As is the case for most MMPs, MT-MMPs contain a C-terminal hemopexin-like domain that confers some degree of substrate specificity (Overall, 2002). However, MT-MMPs differ from the other MMPs by the presence of a C-terminal domain rich in hydrophobic residues that is involved in their association

with the cell membrane. According to the structure of this C-terminal extension (Fig. 1.6), MT-MMPs can be classified into two sub-groups: (1) type I transmembrane proteins, including MT1-, MT2-, MT3- and MT5-MMP, characterized by a long hydrophobic sequence followed by a short cytoplasmic tail and (2) glycosylphosphatidylinositide (GPI)-anchored MT-MMPs (MT4- and MT6-MMP) containing a short hydrophobic signal anchoring to GPI; this sequence is not followed by a cytoplasmic tail. The cytoplasmic tail of type I transmembrane MT-MMPs appears to play an important role in numerous cellular events such as cell signaling, sub-cellular localization, MT-MMP trafficking and dimerization (Itoh and Seiki, 2005; Itoh et al., 2001; Sounni et al., 2004; Uekita et al., 2004; Wang et al., 2004b) (see also section 1.9).

1.6.2 Extracellular matrix substrates of MT-MMPs

The contribution of MT-MMPs to the degradation of a variety of ECM components results in the release of sequestered biologically active proteins and in the regulation of cell migration, growth, differentiation, and survival by controlling cell adhesion and the cytoskeletal organization (Egeblad and Werb, 2002; Zucker et al., 2003). In addition to these matrix substrates, MT-MMPs are also able to cleave other signaling molecules such as cell surface receptors, growth factors, growth factor binding proteins, cytokines/chemokines (Table 1.3). The importance of such MT-MMP proteolytic events in the regulation of cell behavior is described below.

Table 1.3. Extracellular and non-extracellular substrates of MT-MMPs (reproduced from Sounni *et al.*, 2005)

MT-MMPs	ECM substrates	Non-ECM substrates

MT1-MMP	Gelatin, collagens I, II, III, laminin-5, fibronectin, fibrin, proteoglycan	SDF1, MCP-3, proTNFα, IL–8, CTGF, death receptor-6, secretory leukocyte protease inhibitor, CD44, integrin αv subunit, tissue Transglutaminase (tTG), gC1qr, syndecan-1, β glycan, low density lipoprotein receptor related protein (LRP), proMMP-2
MT2-MMP	Collagen I, fibronectin, gelatin, aggrecan, perlecan, laminin-1, tenascin, nidogen	tTG, LRP, proMMP-2
MT3-MMP	Collagen II, III, fibronectin, laminin-1, vitronectin, aggrecan	tTG, CD44, syndecan, LRP, β glycan, α2-macroglobulin, α1-antiproteinase and proMMP-2
MT4-MMP	Fibrinogen, fibrin, gelatin	ProTNFα, aggrecanase-1, LRP
MT5-MMP	Gelatin, fibronectin, chondroitine sulfate, dermatan sulfate	ProMMP-2
MT6-MMP	Gelatin, fibronectin, chondroitine sulfate, dermatan sulfate, collagen type IV, fibrin, fibrinogen	α1-antiproteinase, ProMMP-2

1.6.3 Non extracellular matrix substrates

As introduced above, although MT-MMPs can cleave ECM components, they can also cleave several soluble, cell surface and pericellular proteins (Table 1.3), regulating cell behavior in tumor metastasis and angiogenesis. These regulatory mechanisms include (1) alteration of cell–cell interactions, cell–matrix interactions, (2) release, activation or inactivation of autocrine, or paracrine signaling molecules, (3) shedding or activation of surface receptors and (4) activation of intracellular signaling pathways (Fig. 1.7).

For example, cell surface-associated MMP-2, MMP-9 and MMP-13 can activate latent transforming growth factor-beta (TGFβ) (Egeblad and Werb, 2002)

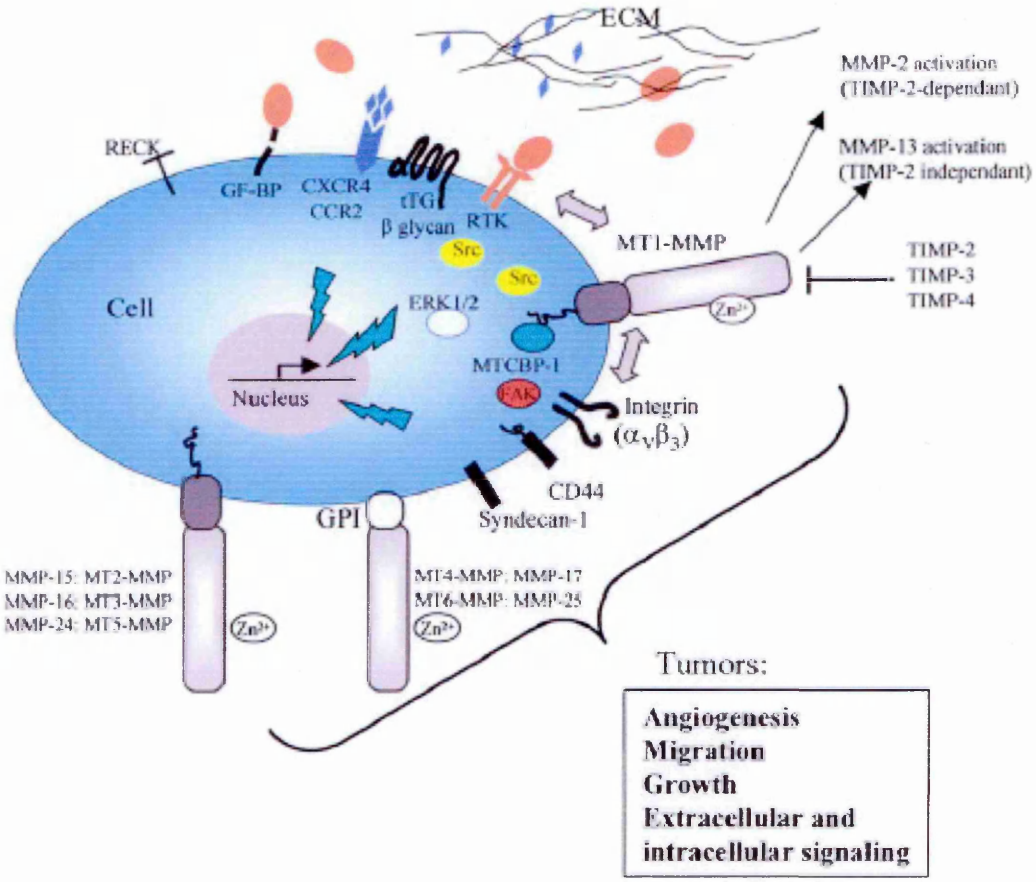


Fig. 1.7. Functions of MT-MMPs in cancer progression associated with their catalytic activities (reproduced from Sounni et al., 2005). The membrane type-matrix metalloproteinases (MT-MMPs) promote cancer progression through different mechanisms: (1) by activating soluble proMMPs, (2) by shedding transmembrane precursors of growth factors, and growth factor binding proteins (GF-BP)), (3) by degrading the extracellular matrix (ECM), facilitating cell migration and the release of growth factors and/or cytokines, (4) by interacting and/or activating receptors and cell surface molecules, (5) by inducing signal transduction and thereby regulating gene expression, (6) by interacting with intracellular molecules such as MTCBP-1. TIMPs and RECK inhibit the activity of MT-MMPs. RTK, receptor tyrosin Kinase; CCR2 and CXCR4, chemokines receptors for MCP-3 and SDF-1, respectively; ERK, extracellular regulated kinase; FAK, focal adhesion kinase; MTCBP-1, MT1-MMP cytoplasmic tail binding protein-1; tTG, tissue transglutaminase; GF-BP, growth factor binding proteins; RECK, Reversion inducing cystein-rich protein with Kasal motifs.

and MT1-MMP modulates the bioavailability of TGFβ through different mechanisms by: (1) activating MMP-13 and MMP-2, (2) releasing active TGFβ from cell surface complexes involving αvβ8 integrin, and (3) releasing a membrane-anchored proteoglycan that binds TGFβ (Velasco-Loyden et al., 2004).

Recently, the list of non-matrix substrates of MT-MMPs has been completed by cytokines and chemokines (Table 1.3). Monocyte chemoattractant protein-3 (MCP-3) is inactivated upon cleavage by MMP-2. Inactivated MCP-3 molecules can also efficiently be generated by a recombinant MT1-MMP and other soluble MMPs such as MMP-1, MMP-3 and MMP-13 (McQuibban et al., 2002). These data provide a new mechanism by which MT-MMPs could control inflammatory response in cancer and other diseases.

1.6.3.1 Role of MT-MMPs in activation of proMMPs

MT-MMPs also control the pericellular activation of proMMPs. The major physiological activators of proMMP-2 (gelatinase A) are members of the MT-MMP sub-group. The activation of proMMP-2 by MT1-MMP involves the action of TIMP-2 (Strongin et al., 1995). TIMP-2 belongs to the family of tissue inhibitors of metalloproteinases (TIMPs) that will be described in section 1.7.3. The currently accepted model involves the stoichiometric binding of TIMP-2 to the MT1-MMP catalytic domain on the cell surface, followed by the binding of C-terminal hemopexin-like domain of proMMP-2 to the C-terminus of TIMP-2 (Seiki, 1999; Seiki et al., 2003) (for more details on MT1-MMP-dependent MMP-2 activation, see section 1.8.3.4).

MT2-, MT5- and MT6-MMP can also activate proMMP-2, but the involvement of TIMP-2 in the activation process is not clear (Morrison et al., 2001). MMP-2 activation by MT1-MMP through a TIMP-2 independent process in endothelial cells can be promoted by αvβ3 integrin (Brooks et al., 1996) or claudin, a tight junction protein (Miyamori et al., 2001). It has recently been

reported (Zhao et al., 2004) that proMMP-2 activation by MT3-MMP also involves formation of a ternary complex on the cell surface. ProMMP-2 can assemble trimolecular complexes with the catalytic domain of MT3-MMP and TIMP-2 or TIMP-3. Based on affinity chromatography experiments, it appears that TIMP-3 binds more efficiently to MT3-MMP than to MT1-MMP and enhances the activation of proMMP-2 by MT3-MMP but not by MT1-MMP (Zhao et al., 2004). Finally, comparison of the activation of proMMP-2 by cells expressing MT1-, MT2- or MT3-MMP, demonstrated that both MT2-MMP and MT3-MMP are less efficient than MT1-MMP (Sounni and Noel, 2005). The catalytic domain of MT6-MMP can activate proMMP-2 and generates a higher ratio of mature/intermediate forms of MMP-2 than MT5-MMP (Nie and Pei, 2003). At variance with the other MT-MMPs, MT4-MMP is an inefficient proMMP-2 activator (English et al., 2001a; English et al., 2000).

MT1-MMP also contributes to the activation of procollagenase-3 (MMP-13), directly, or indirectly via MMP-2 activation (Knauper et al., 1996). ProMMP-13 activation by MT1-MMP does not involve the establishment of a trimolecular complex between proMMP-13, MT1-MMP and TIMP-2 (Knauper et al., 2002). The role of MT-MMPs in proMMP activation was extended by recent studies (Toth et al., 2003) reporting that proMMP-9 activation is initiated by MT1-MMP and then mediated by MMP-2 or MMP-3. The activation of proMMP-9 by MT1-MMP/MMP-2 axis is regulated by the concentration of TIMP-2 (Toth et al., 2003).

Altogether, these data demonstrate that MT-MMPs participate in a zymogen activation cascade leading to an amplification of proteolysis, in proximity to the cell surface. This pericellular activation of MMPs acts in a concerted manner with the cascade of protease activation involving the plasminogen/plasmin activator system leading also to proMMP activation.

1.7 Regulation of MMP activity

The proteolytic activity of MMPs is regulated at two main levels: 1) transcription and 2) proenzyme activation. Additional mechanisms by which MMP activity is fine-tuned involve regulation of mRNA stability, translational efficiency, enzyme compartmentalization, secretion, cell-surface recruitment, shedding, oligomerization, cellular uptake and internalization, substrate targeting, autolysis and finally, enzyme inhibition (Fig. 1.8).

These mechanisms operate coordinately to ensure that MMP expression and activity are confined to those sites and conditions in which proteolytic activity is necessary. Malignant tumours, however, have developed strategies to circumvent these regulatory mechanisms, and to generate the uncontrolled proteolytic activity that accompanies cancer development and metastasis (Overall and Lopez-Otin, 2002).

1.7.1 Gene expression

Regulatory mechanisms of MMP gene transcription exist for cell-type and stimulus specificity (Birkedal-Hansen et al., 1993). Many of the MMPs are expressed only in a few cell types or tissues, suggesting the presence of cell or tissue-specific promoters, enhancers and/or silencers (Harendza et al., 1995). Most MMP genes are not expressed in normal adult tissues or by unstimulated cells *in vitro*, but their expression is markedly induced by growth factors, pro-inflammatory cytokines, and phorbol esters (Westermarck and Kahari, 1999). In contrast, MT1-MMP is constitutively expressed *in vitro* by different cell types including fibroblasts, endothelial cells, and smooth muscle cells, and its expression is only modestly enhanced by the tumor promotor PMA, TNF-α, and the lectin concanavalin A (ConA), not affected by TGF-β, and decreased by dexamethasone (Lohi and Keski-Oja, 1995). In addition, the mRNAs for all other MT-MMPs are detectable by Northern blot analysis in normal human tissues.

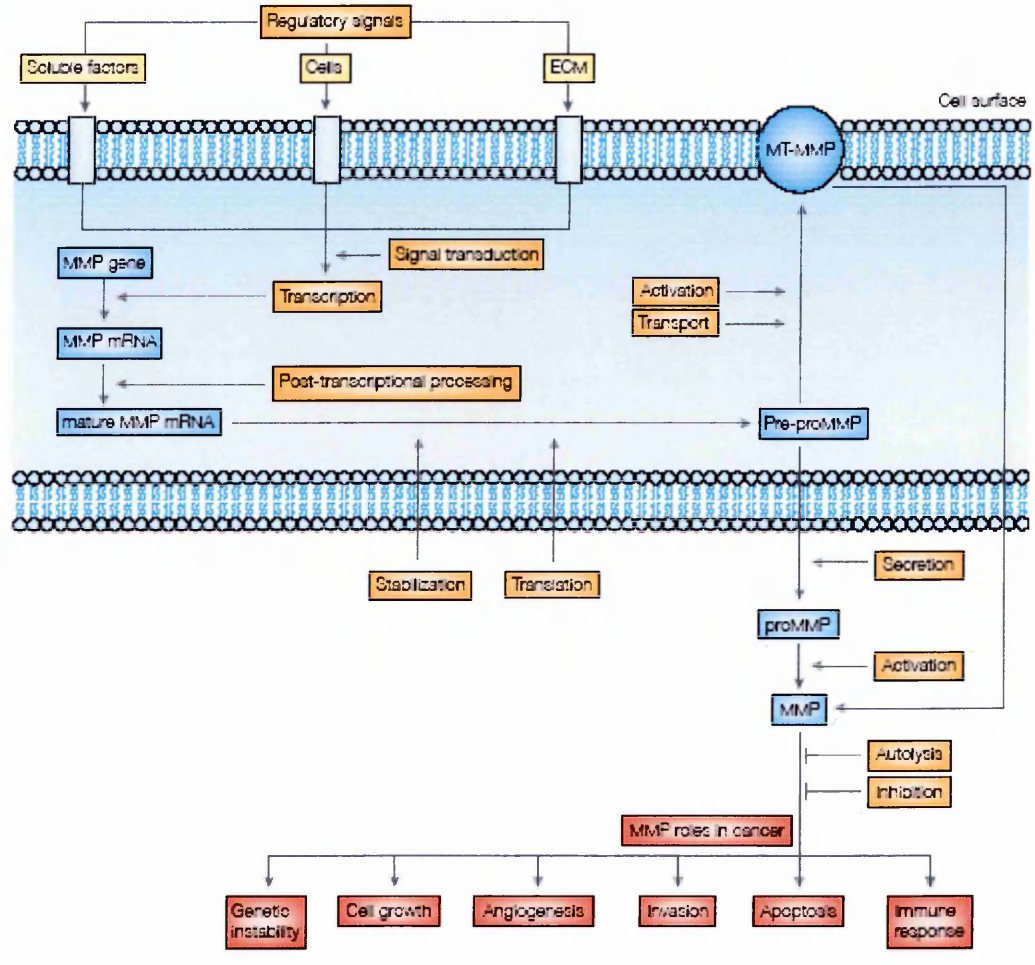


Fig. 1.8. Levels of regulation of MMP expression and activity (reproduced from Overall and Lopez-Otin, 2002). Diverse regulatory signals, such as soluble factors, extracellular-matrix–cell interactions (ECM) or cell–cell contacts (cells) interact with specific receptors at the cell surface and initiate a cascade of events that lead to the generation of functional matrix metalloproteinases (MMPs), which are localized to the cell surface (MT-MMPs) or secreted to the extracellular medium (proMMP). ProMMPs are activated by different events. These active MMPs are involved in a number of processes that promote cancer development (red boxes), including promoting genetic instability, cell growth, angiogenesis and invasion. They also interfere with apoptosis induction and the host antitumour immune response. MMP autolysis or inhibitors can interfere with the induction of these cellular effects. The levels of MMP regulation that might be therapeutically targeted are shown in orange boxes, and include cell responses to regulatory signals, signal transduction, transcription induction, post-transcriptional processing, MMP activation and transport, and secretion.

MMP-2 is also constitutively expressed in most fibroblastic cells, and its expression is enhanced by TGF-β but not by phorbol esters (Collier et al., 1988). Both MT1-MMP and MMP-2 promoters have Sp1 (transcription factor specificity protein 1) binding sites that are important for basal promoter activity.

In endothelial cells cultured in a collagen matrix, enhanced MT1-MMP transcription is driven by increased binding of Egr-1 (early growth response transcription factor-1) - a zinc finger transcription factor critical for coupling extracellular signals to changes in cellular gene expression - to the MT1-MMP promoter (Haas et al., 1999). The MT1-MMP promoter features consensus binding sites for Sp1 and Egr-1, whereas the MMP-2 promoter does not contain Egr-1 binding sites, thus allowing cells to specifically upregulate MT1-MMP. Interestingly, the induction of Egr-1 expression occurs in response to potential angiogenesis initiators such as wound formation, mechanical stress, and fluid shear stress (Khachigian et al., 1996; Silverman and Collins, 1999). Other details about transcriptional regulation of MT1-MMP gene expression will be described in section 1.8.3.1.

1.7.2 Zymogen activation

All MMPs are synthesized as latent zymogens containing about 10-kDa amino terminal globular prodomain that protrudes into the active site of the enzyme and maintains its latency. The propeptide serves to maintain proMMPs in an inactive state (Becker et al., 1995; Morgunova et al., 1999). A cysteine-zinc bond links the unpaired cysteine residue in the prodomain to the catalytic zinc ion preventing the access of a water molecule that is necessary for catalysis (Bode et al., 1999b). The sequence surrounding the cysteine in the prodomain, (K)PRCGV/NPD(V), and the zinc binding sequence in the catalytic domain, HEXGHXXGXXH, are the two best-conserved sequences in MMPs (Bode et al., 1999a). Activation of the soluble proMMPs can be accomplished *in vitro* by detergents, oxidants, mercurial compounds, sulfhydryl reagents, and acidic pH, all

of which expose the prodomain cysteine by conformational change, and cause the disruption of Cys-Zinc bond, also called "cysteine switch" (Springman et al., 1990). In addition, cleavage of the prodomain by various serine-, cysteine-, and metalloproteinases leads to unfolding and exposure of the remaining portion of the propeptide and disruption of the cysteine-zinc bond. The initial cleavages usually occur usually in the flexible, exposed αI-αII loop and is followed by a series of (autocatalytic) cleavages leading to the generation of the mature enzyme.

In most cases the proteolytic activation of proMMP occurs extracellularly because several soluble enzymes can activate most secreted MMPs (Mignatti and Rifkin, 1993; Mignatti and Rifkin, 2000). For instance, the lack of activation of many MMPs (MMP-3, MMP-9, MMP-12, and MMP-13) in macrophages of uPA-deficient mice suggests an important role of the cell surface plasminogen activator/plasmin system on the activation of some secreted MMPs *in vivo* (Carmeliet et al., 1997).

The characterization of constitutive intracellular activation of prostromelysin-3 (MMP-11) by the Golgi-associated endopeptidase furin unveiled a novel mechanism for the activation of MMPs (Pei and Weiss, 1995). A basic sequence RXRXKR present in the prodomain was found to signal the cleavage as shown by site-directed mutagenesis. All MT-MMPs contain similar basic recognition sequences (RXK/RR) at the C-terminal end of the prodomain, and these MMPs also appear to be activated during transport to the cell surface. This aspect will be described in-depth in section 1.8.3.2 regarding the activation of MT1-MMP because its characterization is one of the main topics of my thesis.

1.7.3 Metalloproteinase inhibitors

Endogenous inhibitors such as the tissue inhibitors of metalloproteinases (TIMPs) and serum α2-macroglobulin (α2M) can inhibit the activated MMPs *in vivo*. α2M is an abundant serum inhibitor effective on many types of proteinases

(Birkedal-Hansen et al., 1989; Sottrup-Jensen et al., 1989). In addition, thrombospondins can inhibit MMP-2 and -9 activation and induce their clearance through scavenger receptor-mediated endocytosis (Bein and Simons, 2000). New insight into the regulation of cell surface proteolysis was provided by the characterization of a GPI-anchored membrane glycoprotein, RECK (revision-inducing cysteine-rich protein with kazal motifs), which was shown to down regulate MT1-MMP, MMP-2 and MMP-9 function and tumor angiogenesis and metastasis (Oh et al., 2001; Takahashi et al., 1998).

The TIMPs are considered to be the key local MMP regulators in tissues. The TIMP family consists of four human TIMPs, TIMP-1, -2, -3, and -4, which share 30-50% sequence identity (Docherty et al., 1985; Greene et al., 1996; Silbiger et al., 1994; Stetler-Stevenson et al., 1989). They are composed of N- and C-terminal domains both of which are stabilized by three disulfide bonds between six conserved cysteine residues (Bode et al., 1999a; Fernandez-Catalan et al., 1998; Tuuttila et al., 1998). TIMPs also have differential abilities to form complexes with progelatinases through interactions between their C-terminal domains and the C-terminal hemopexin domains of progelatinases (Henriet et al., 1999).

All TIMPs can inhibit most MMPs by tight non-covalent binding to their active site in a 1:1 stoichiometric ratio (Gomez et al., 1997; Howard and Banda, 1991). MT-MMPs are an exception as TIMP-1 is a poor inhibitor of MT1-, MT2-, MT3-, and MT-5-MMP (Shimada et al., 1999). In contrast, MT4- and MT6-MMP are effectively inhibited by TIMP-1 (English et al., 2001b; Kolkenbrock et al., 1999).

TIMPs have been thought to be mainly regulated at the level of gene expression. Various cultured cells constitutively express TIMP-2, whereas several growth factors/cytokines and chemicals upregulate the expression of TIMP-1 (Gomez et al., 1997). TIMP-3 expression is induced in cultured cells by stimulation with serum, EGF, and TGF-β. Unlike other TIMPs, TIMP-3 is

insoluble and binds to ECM both *in vitro* and *in vivo*. TIMPs are generally very stable, although they may be inactivated by limited proteolysis by serine proteinases such as trypsin and elastase (Itoh and Nagase, 1995; Okada et al., 1988). Maquoi *et al.* demonstrated that internalization through an MT1-MMP dependent mechanism and subsequent degradation downregulate TIMP-2 in tumor cell cultures (Maquoi et al., 2000).

Due to their ability to inhibit proteolytic degradation required for cell invasion, TIMPs have been indicated as negative regulators of processes like angiogenesis and cancer invasion (Gomez et al., 1997). Indeed, TIMP-1 and TIMP-2 inhibit endothelial and vascular smooth muscle cell migration (Cheng et al., 1998; Fernandez et al., 1999), while TIMP-2 reduces melanoma cell invasion, tumor growth and angiogenesis *in vivo* (Valente et al., 1998). Adenovirus-mediated gene delivery of TIMP-3 inhibits melanoma cell invasion (Ahonen et al., 1998) and overexpression of TIMP-4 inhibits invasion of breast cancer cells *in vitro* and tumor growth and metastasis *in vivo* (Wang et al., 1997). On the other hand, TIMP-1, -2, and -3 stimulate the growth of several cell types (Henriet et al., 1999). TIMP-2 can suppress EGF-mediated mitogenic signaling (Hoegy et al., 2001), and TIMP-3 can induce apoptosis of normal and malignant cells (Ahonen et al., 1998; Baker et al., 1998). Recent data also suggest the involvement of TIMPs in other invasive processes such as implantation (Henriet et al., 1999).

1.8 MT1-MMP

1.8.1 Background

MT1-MMP was initially observed as a membrane-associated MMP-2 activator (Overall et al., 1989). Subsequently, in 1994, Sato *et al.* discovered the first membrane-type MMP, now known as MT1-MMP, and identified it as a cell-surface proMMP-2 activator (Sato et al., 1994). Since its discovery, MT1-MMP

has been extensively investigated and is now one of the best characterized MMPs. MT1-MMP has been implicated in various physiological and pathological processes such as wound healing, bone development, angiogenesis, inflammation, rheumatoid arthritis, atherosclerosis, and cancer invasion and metastasis (Itoh and Seiki, 2005).

Ablation of the gene encoding MT1-MMP causes craniofacial dysmorphism, arthritis, osteopenia, dwarfism and fibrosis of soft tissues in mice (Holmbeck et al., 1999). This is the most significant phenotype among the MMP knockout mice, and suggests that the activity of MT1-MMP, differently from other MMPs, cannot be substituted by any of the other members of the family. MT1-MMP is highly expressed in different cancers, and its overexpression promotes migration, invasion and metastasis of cancer cells *in vitro* as well as *in vivo* (Seiki, 2003). The enzyme has also been shown to be essential for angiogenesis (Hiraoka et al., 1998; Zhou et al., 2000). Furthermore, Hotary *et al.* recently reported that expression of MT1-MMP is crucial for cancer-cell growth in a 3D collagen-based matrix, suggesting that MT1-MMP has important roles not only in cancer invasion but in tumor progression overall (Hotary et al., 2003).

MT1-MMP degrades various extracellular matrix (ECM) macromolecules including collagen I, II and III, fibronectin, laminin 1 and 5, fibrin, and aggrecan (Itoh and Seiki, 2004). On the cell surface, MT1-MMP also activates other members of the MMP family, for example, proMMP-2 and proMMP-13, creating a wider proteolytic repertoire on the cell surface. MT1-MMP cleaves CD44, which promotes cell migration; it processes αv integrin to the mature form; it degrades transglutaminase, modifying the attachment of the cells to fibronectin (for a review, Itoh and Seiki, 2005); and also cleaves low-density lipoprotein receptor-related protein (LRP), which is a scavenger receptor for proteinases in the extracellular milieu (Rozanov et al., 2004b). In addition, cleavage of the laminin 5γ chain by MT1-MMP also stimulates epithelial-cell motility (Koshikawa et al., 2000). Because MT1-MMP acts on the cell surface, it

modifies the immediate environment of the cell resulting in alteration of signaling from the ECM or adhesion molecules, thereby influencing cellular functions. For MT1-MMP to undertake these activities effectively, negative and positive regulation of the enzyme at different levels is essential.

Considering the biological relevance of this enzyme and the fact that it is the main topic of my thesis, the remaining part of the introduction will describe how MT1-MMP affects cellular function by changing the microenvironment, and the many ways in which cells can regulate the activity of this enzyme.

1.8.2 Modulation of cell functions by MT1-MMP

Expression of MT1-MMP on the cell surface, together with the MMPs it activates, creates wide proteolytic repertoire in the pericellular space, and allows for efficient proteolytic changes to the cellular microenvironment which can result in modification of different cellular functions (Fig. 1.9).

1.8.2.1 MT1-MMP promotes cell invasion

When a cell migrates in tissue, the ECM is a physical barrier and needs to be degraded to clear a path (Murphy and Gavrilovic, 1999). MMP-2 degrades type IV collagen, a major component of the basement membrane. As cancer cells need to traverse the basement membrane to achieve invasion and metastasis, the activation of proMMP-2 by MT1-MMP is considered to be a critical step (Seiki, 2003; Stetler-Stevenson et al., 1993). Overexpression of MT1-MMP enhances cellular invasiveness in *in vitro* invasion experiments with Matrigel, a reconstituted basement membrane (Sato et al., 1994) and in an *in vivo* lung metastasis assay, which requires degradation of real basement membrane (Tsunezuka et al., 1996). Furthermore, MT1-MMP gene silencing using double

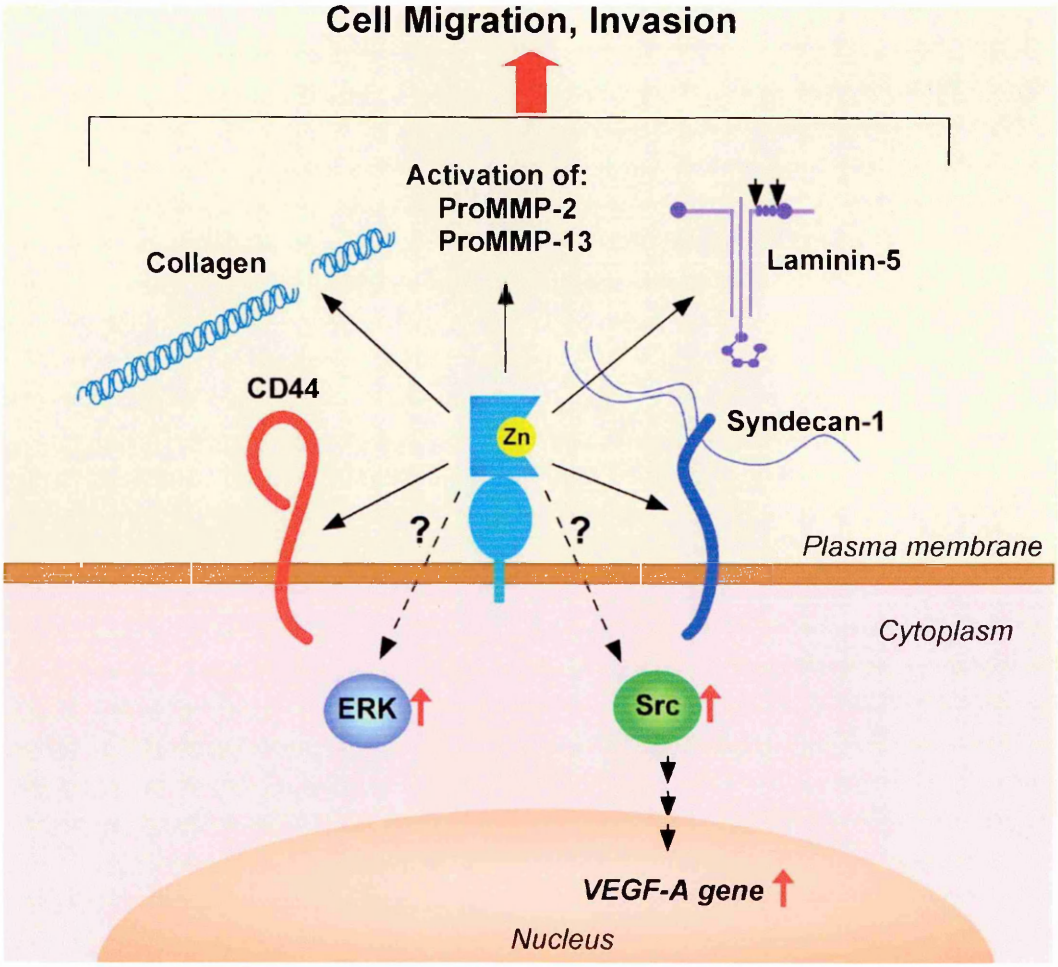


Fig. 1.9. Biological functions of MT1-MMP which promote cell migration and invasion (reproduced from Itoh and Seiki, 2005). MT1-MMP sheds CD44 and syndecan-1, degrades collagens and other matrix components, releases EGF-like repeats from laminin 5, activates proMMP-2 and proMMP-13, upregulates ERK, and upregulates VEGF-A expression through Src. All of these activities result in increased cell migration and invasion in tissue.

stranded RNA interference in cancer cells also showed that downregulation of MT1-MMP only is sufficient to inhibit Matrigel invasion of cancer cells significantly (Ueda et al., 2003). These findings strongly suggest that MT1-MMP is indeed a critical part of the invasion machinery.

1.8.2.2 MT1-MMP is a critical collagen environment modulator

An important function of MT1-MMP is its collagen degrading activity. All collagenolytic MMPs cleave interstitial collagen at a specific site, 3/4 away from the N-terminus (see Fig. 1.4), which then initiates denaturation of triple helical collagen into gelatin, rendering it susceptible to many different proteinases (Visse and Nagase, 2003). Among the collagenases, MT1-MMP is unique in that it is a membrane-tethered collagenase (Holmbeck et al., 2004). The importance of MT1-MMP as a collagenase is particularly highlighted by the phenotype of MT1-MMP gene knockout (KO) mice (Holmbeck et al., 1999; Zhou et al., 2000). The neonatal KO mice are indistinguishable from heterozygous and wild-type littermates, but at 5 days of age growth impairment becomes apparent (Holmbeck et al., 1999; Zhou et al., 2000). KO mice develop severe fibrosis in periskeletal soft tissue, and show delayed ossification of bone, and die around 7-12 weeks of age (Holmbeck et al., 1999; Zhou et al., 2000). Fibroblasts derived from KO mice lack the ability to degrade type I collagen (Holmbeck et al., 1999).

Detailed analyses suggest that the bone phenotype of the KO mice may in part result from the lack of cellular collagen degradation by osteocytes (Holmbeck et al., 2005). This suggests that osteocytes were not able to create the right ECM environment for osteocytogenesis. No other MMP gene KO mice have shown such drastic phenotypes and many events including long bone growth, soft tissue organization, molar root formation and eruption, and cartilage remodeling in the skull are found to be MT1-MMP-dependent (Holmbeck et al., 2004). These findings strongly suggest that MT1-MMP is an essential cellular collagenase important for organizing the ECM microenvironment, which cannot be substituted

for by any other MMP during development.

MT1-MMP is also an important collagenase during migration of cells in a collagen matrix; only MT1-MMP can promote cellular invasiveness into collagen I matrix in epithelial cells and fibroblasts (Hotary et al., 2000; Sabeh et al., 2004). Other soluble collagenase MMPs, such as MMP-1, MMP-2, MMP-8, and MMP-13, fail to promote invasion even though they are secreted as an active form under the experimental conditions used (Hotary et al., 2000; Sabeh et al., 2004). For MT1-MMP to promote collagen invasion, the enzyme needs to be membrane-bound since a soluble form of MT1-MMP lacking the transmembrane and cytoplasmic domains was not able to do so (Hotary et al., 2000; Sabeh et al., 2004). Thus, MT1-MMP as a membrane-tethered collagenase plays a unique role.

Interestingly, pericellular collagen degradation is essential not only for invasion, but also for tumor cells to grow within a collagen-based 3D matrix (Hotary et al., 2003). Again, MT1-MMP was found to play a critical role. The effect of MT1-MMP is specific to 3D cultures, suggesting that it is acting although pericellular degradation of collagen matrix. Soluble collagenase MMPs (MMP-1, MMP-2, and MMP-13) cannot substitute for MT1-MMP, and without MT1-MMP tumor cells cannot proliferate (Hotary et al., 2003). Though the role of MT1-MMP in metastasis was originally ascribed to the cancer cell invasion step, these findings suggest that MT1-MMP may also play a role in promoting tumor growth.

1.8.2.3 MT1-MMP stimulates cell motility

Initially, the enhanced invasiveness caused by MT1-MMP expression was thought to be due to enhanced pericellular ECM degradation. It has now become evident that path clearance is not the only mechanism. It was found that shedding of CD44 from the cell surface by MT1-MMP enhances cell migration on a hyaluronan-based 2D matrix (Kajita et al., 2001). CD44 is a widely expressed major hyaluronan receptor, which interacts with the cytoskeleton though its

cytoplasmic domain. The exact mechanism behind the MT1-MMP/CD44-mediated promotion of cell migration is not clear. MT1-MMP may regulate the adhesion properties of lamellipodia through CD44 shedding or it may modulate signals mediated by CD44. A similar observation was made with syndecan-1 (Endo et al., 2003). MT1-MMP causes release of syndecan-1 and this shedding is required for efficient cell migration on collagen. Again the underlying mechanism is not clear. Syndecan-1 is known to associate with, and stabilize, αvβ3 integrin, promoting cell spreading (Beauvais et al., 2004). It is possible that shedding of syndecan-1 associated with αvβ3 affects integrin signaling. Expression of MT1-MMP is reported to activate extracellular signal-regulated protein kinase (ERK) and ERK activation is shown to be essential for MT1-MMP-dependent cell migration (Gingras et al., 2001; Takino et al., 2004). It is not clear how MT1-MMP activates ERK, but this may be a part of the mechanism(s) of CD44-mediated cell migration. Elucidating this mechanism may be one of the keys to understanding how MT1-MMP promotes cell migration (see Chapter 5).

Another mechanism for MT1-MMP-mediated stimulation of cell motility is through the cleavage of laminin 5 (Koshikawa et al., 2005), a major component of basement membrane. Indeed, MT1-MMP cleaves the γ2 chain of laminin 5, releasing EGF-like domains, which bind the EGF receptor and stimulate epithelial cell motility (Schenk and Quaranta, 2003). Cleavage of the γ2 chain of laminin 5 by MT1-MMP may be tissue-specific as in keratinocytes astacin-like metalloproteinases, bone morphogenic protein-1 (BMP-1), and its variant forms (tolloid metalloproteinases), but not MT1-MMP, are reportedly responsible for γ2 chain cleavage (Veitch et al., 2003). However, it is not clear if this cleavage of laminin γ2 chain by BMP-1 affects keratinocyte migration. It has also been shown that the β3 chain of human laminin 5 is cleaved by MT1-MMP (Udayakumar et al., 2003). Interestingly, the cleavage product is shown to stimulate prostate cancer cell motility (Udayakumar et al., 2003), but the mechanism remains to be

determined.

1.8.2.4 MT1-MMP promotes angiogenesis

Angiogenesis is a formation of new vessels from existing vessel and provides a mechanism to establish a blood supply. It is fundamental for wound healing, tumor growth, and progression of rheumatoid arthritis (Sivakumar et al., 2004). Angiogenesis itself is a process of cellular invasion by endothelial cells: they need to detach from neighboring cells, invade into stromal tissue, proliferate, and generate a tube structure. During this process, endothelial cells need to degrade basal lamina, fibrin, and collagen enriched stromal tissue. Previous reports have shown that MMP-2 (Brooks et al., 1998), MMP-9 (Bergers et al., 2000), and their cognate cell surface receptors such as αvβ3 integrin (Brooks et al., 1998; Silletti et al., 2001) and CD44 (Yu and Stamenkovic, 1999) play a critical role in this process, but Chun *et al.* recently reported that although plasmin, MMP-2, MMP-9, αvβ3 integrin, and CD44 are not essential for angiogenesis, MT1-MMP is (Chun et al., 2004). Tissues from mice lacking the former genes generated normal neovessels in type I collagen in an ex vivo model, but MT1-MMP-less tissue failed to support angiogenesis (Chun et al., 2004). This observation is specific for collagen matrix; in fact, in a fibrin matrix, MT1-MMP KO mice tissue supported normal neovessel formation. The fact that neovessel formation in fibrin gel is inhibited by TIMP-2 but not by TIMP-1, and the observation that MT2-MMP and MT3-MMP gene expression was upregulated during tube formation by human umbilical vein endothelial cells in fibrin gels (Lafleur et al., 2002) suggests that these MT-MMPs may compensate for MT1-MMP. MT1-MMP also promotes angiogenesis by means other than stimulation of endothelial cell invasion. Expression of MT1-MMP in tumors stimulates angiogenesis *in vivo* by stimulating VEGF synthesis by the tumor cells (Deryugina et al., 2002; Sounni et al., 2002). MT1-MMP specifically upregulates VEGF-A gene expression, and this is mediated by src tyrosine kinase (Sounni et

al., 2004). This event requires proteolytic activity of MT1-MMP and is dependent on the cytoplasmic domain (Sounni et al., 2004). The mechanism behind this is not clear, but MT1-MMP activity on the cell surface presumably modifies the immediate microenvironment, which triggers a cell-signaling pathway to produce VEGF-A.

Taken together, MT1-MMP is an important pericellular microenvironment modifier which affects cell functions and promotes invasion in tissue.

1.8.3 Regulation of MT1-MMP

1.8.3.1 Transcriptional regulation of MT1-MMP gene expression

As already mentioned in section 1.7.1, MT1-MMP gene expression can be regulated by cytoskeleton-ECM interactions. It is induced in fibroblasts and endothelial cells by culture in three dimensional collagen matrixes, by mechanical stretching, and by treatment of the cells with the cytoskeleton disrupting agent cytochalasin D (reviewed in Overall and Lopez-Otin, 2002). Therefore, signaling pathways initiated through collagen binding β1 integrins may regulate MT1-MMP expression (Haas and Madri, 1999). Interestingly, the induction of MT1-MMP occurs when the cells are cultured on or within a type I collagen gel, but does not occur when cells are grown on a thin coating of type I collagen (Azzam and Thompson, 1992; Haas et al., 1998; Tomasek et al., 1997). Recently, it was also demonstrated that collagen matrix provides cells with various signals for growth, survival, differentiation, migration and so on. Extra-cellular signal-regulated kinase (ERK) is activated in cells cultured either with a type I collagen matrix or on a collagen sheet, through interaction with integrins. ERK activated by collagen culture of cells in turn stimulates MT1-MMP expression (Takino et al., 2004). Activation of ERK is not maintained in the presence of MMP inhibitors, however.

Thus, MT1-MMP functions in a positive feedback loop to induce sustained ERK activation and subsequent MT1-MMP accumulation, which collectively promotes cell migration on collagen (Sato et al., 2005).

MT1-MMP expression levels are also higher in fibroblasts cultured on a floating or stress-relaxed collagen lattice compared with the expression levels in actin stress-fiber containing fibroblasts cultured in stabilized lattices (Tomasek et al., 1997). Therefore, the induction of MT1-MMP expression could involve, in addition to ligand-specific integrin interactions, alterations in mechanical force and in the assembly of the actin cytoskeleton.

Cytokines also appear to regulate MT1-MMP expression during inflammation. Although TNF-α alone has little effect on MT1-MMP gene expression in cultured fibroblasts (Han et al., 2001), activation of NF-kB signaling synergistically by TNF-αand type I collagen induces MT1-MMP expression in skin fibroblasts (Han et al., 2001). TNF-α, IL-1α, and IL-1β also up regulate MT1-MMP gene expression in vascular endothelial cells (Rajavashisth et al., 1999). In human monocytes MT1-MMP expression is induced by lipopolysaccharide through the prostaglandin-cAMP dependent pathway (Shankavaram et al., 2001).

1.8.3.2 MT1-MMP modular domain structure and its proteolytic processing

MT1-MMP is produced as a 63 kDa protein (also referred to as proMT1-MMP) and shares a common domain structure with other MMP family members, including a signal peptide (M1-S33), a propeptide domain (P34-R111), a catalytic domain (Y112-G285), a hinge region (E286-I318), a hemopexin domain (C319-C508), a linker region (P509-S538), a transmembrane domain (A539-F562), and a cytoplasmic tail (R563-V583) (Fig. 1.10). Three peculiar insertions, not found in most secreted MMPs, may determine the many distinct features of MT1-MMP:

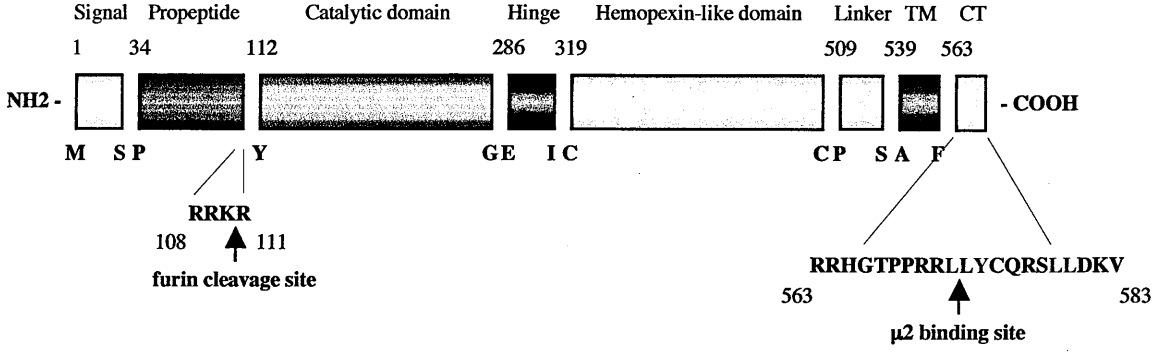


Fig. 1.10. MT1-MMP modular domain structure. MT1-MMP is produced as a 63 kDa protein and shares a common domain structure with other MMP family members, including a signal peptide, a propeptide domain, a catalytic domain, a hinge region, a hemopexin-like domain, a linker region, a transmembrane domain (TM) and a cytoplasmic tail (CT).

1) an 11-aa insertion between the propeptide and the catalytic domain that contains an RRKR sequence, a recognition motif for cleavage by subtilisin-like Golgi associated proteinases (present also in MMP-11, MMP-23 and MMP-28); 2) an 8-aa insertion in the N-terminal part of the catalytic domain (MT-loop); and 3) a C-terminal insertion containing a stretch of 24-aa hydrophobic sequence (a transmembrane domain) followed by a 20-aa cytoplasmic domain. The crystal structure of MT1-MMP catalytic domain in complex with TIMP-2 has been characterized (Fernandez-Catalan et al., 1998): it consists of a five-stranded β sheet and three α helixes characteristic for the MMP fold. The structure differs from a classical MMP fold mostly by the presence of two large insertions distals to the active site cleft: the long flexible loop between βII and βIII strands known as the "MT-MMP loop" and the elongated loop between βV strand and αB helix.

As mentioned, the 63 kDa proMT1-MMP contains a basic recognition sequence (RRKR) at the C-terminal end of the prodomain that appears to be processed during its transport to the cell surface to produce a 60 kDa MT1-MMP form. It has been shown that co-expressed furin, a subtilisin-like proteinase, activates a truncated MT1-MMP mutant lacking the transmembrane and cytoplasmic domains; furthermore, an irreversible inhibitor of furin, the Pittsburgh mutant of α1-antitrypsin, prevents MT1-MMP activation (Pei and Weiss, 1996; Sato et al., 1996). The N-terminus of the furin-activated MT1-MMP mutant is identical to the endogenous MT1-MMP purified from HT-1080 fibrosarcoma cells (Strongin et al., 1995). In addition, treatment of human fibroblasts, fibrosarcoma cells as well as several melanoma cells with the synthetic furin inhibitor Dec-RVKR-CH_2Cl inhibits full-length MT1-MMP processing and MMP-2 activation (Kurschat et al., 1999; Maquoi et al., 1998; Sato et al., 1999). This evidence would support the idea that furin is the most likely candidate for MT1-MMP activation.

Yana and Weiss (Yana and Weiss, 2000), on the other hand, described furin-independent pathways for full-length MT1-MMP processing that are

affected by the membrane tethering of the enzyme. These alternative pathways were described by using cell types expressing different furin-like proprotein convertases.

Conversely, Cao and Zucker (Cao et al., 1998; Cao et al., 2000; Cao et al., 1996) have questioned the requirement for the proteolytic removal of the prodomain, suggesting that the prodomain is required for binding of TIMP-2 to the catalytic domain as well as for the catalytic activity of MT1-MMP (Cao et al., 2000). A possible explanation for this requirement for the prodomain may be related to a chaperone-like function essential for folding and trafficking of the enzyme (Cao et al., 2000). This function is dependent on the conserved $Y_{42}GYL_{45}$ sequence within the propeptide domain (Pavlaki et al., 2002). The degree of catalytic MMP activity obtained by conformational changes without removal of the prodomain is currently unclear (Bannikov et al., 2002).

The role of furin in MT1-MMP activation as well as the role of the MT1-MMP prodomain in MT1-MMP catalytic activity are still completely debated and for this reason I decided to investigate these issues in my experimental work (see Chapter 3).

It has been also shown that its 60 kDa active form undergoes further processing to a 44-45 kDa species by MMP-2 or MT1-MMP itself (Lehti et al., 1998; Stanton et al., 1998; Toth et al., 2002). This step inactives MT1-MMP by removal of its catalytic domain, and is a downregulatory mechanism. The proteolytic processing of MT1-MMP may be an indication of how active the enzyme is on the cell surface. A high level of 45 kDa form coincides with high proMMP-2 activation whereas no proMMP-2 activation appears to occur when the 45 kDa form is not detected (Lehti et al., 1998; Stanton et al., 1998). Thus, MT1-MMP can either be functionally active, undergoing further processing, or functionally inactive, remaining as an intact mature form. This suggests that there may be a step which regulates its functional activity after expression of the mature enzyme on the cell surface.

In some cells, the whole ectodomain of MT1-MMP was shown to be shed (Harayama et al., 1999; Toth et al., 2002; Toth et al., 2004). Cleavage occurs at the Val524-Ile bond in the linker-2 region and releases functional MT1-MMP from the cell surface (Toth et al., 2004). Although shed MT1-MMP may act as a soluble proteinase in the extracellular milieu, the biological activity of MT1-MMP on cell function would effectively be lost since the enzyme needs to be membrane-tethered to exert these effects (Cao et al., 1995; Hotary et al., 2000; Sabeh et al., 2004). Thus, this can be another aspect of the downregulatory mechanism, although it is not clear how frequently and widely this shedding event actually occurs.

1.8.3.3 Inhibition of MT1-MMP activity

On the cell surface, active MT1-MMP is one of the main factors that influence the cellular microenvironment; its inhibition is thus one of the critical steps to regulate its activity (Fig. 1.11).

MT1-MMP is inhibited by endogenous inhibitors TIMP-2, -3, and -4, but not by TIMP-1 (Bigg et al., 2001; Will et al., 1996). This TIMP-1 insensitive nature is common to all transmembrane type MT-MMPs (e.g., MT2-, MT3-, and MT5-MMPs) and allows them to work under conditions where high levels of TIMP-1 are present. Another inhibitor for MT1-MMP is RECK (reversion-inducing-cysteine rich protein with Kazal motifs), a GPI-anchored glycoprotein (Oh et al., 2001). RECK was originally discovered as a tumor invasion suppressor gene with dual functionality: suppressing MMP-9 expression and inhibiting its enzyme activity (Takahashi et al., 1998). Later, it was also found to inhibit the proteolytic activity of MT1-MMP and MMP-2 (Oh et al., 2001). Mice with a disrupted RECK gene do not survive and show defects in collagen fibrils, the basal lamina, and vascular development, a phenotype that may be the result of excess MMP activities. Interestingly, this phenotype was partially suppressed by an MMP-2 null mutation (Oh et al., 2001).

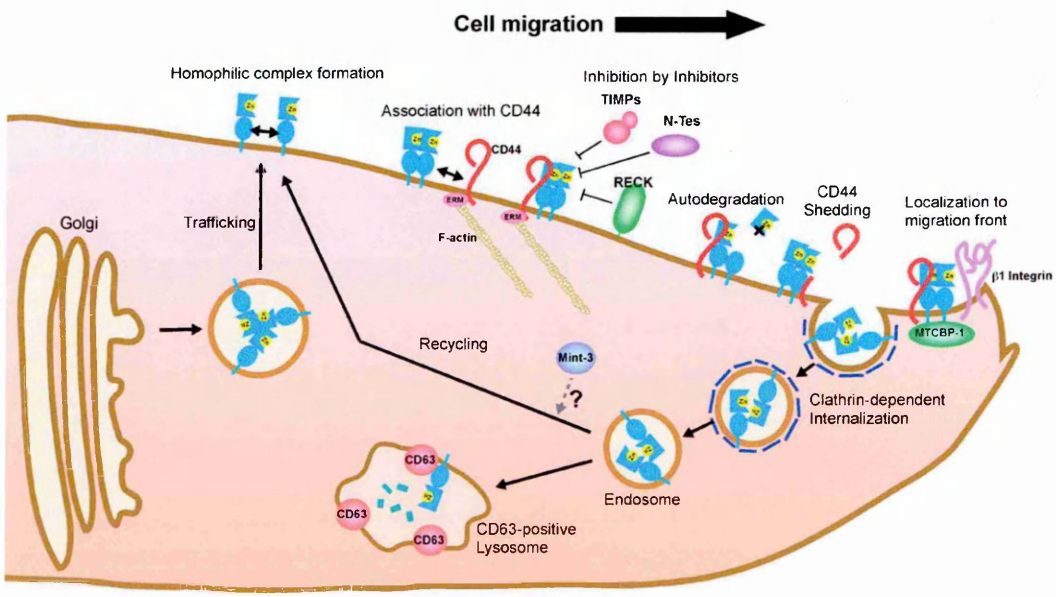


Fig. 1.11. Cellular regulation of MT1-MMP (reproduced from Itoh and Seiki, 2005). MT1-MMP is regulated in a number of ways including: transcription, trafficking to cell surface, homophilic complex formation, association with CD44 followed by localization to migration front, autodegradation, and inhibition by inhibitors such as TIMPs, N-Tes, and RECK. The enzyme is also internalized by caveolae-dependent and clathrin-dependent pathways. The internalized enzyme can be recycled back to the cell surface or degraded in CD63-positive lysosomes. MT1-MMP at the migration front can colocalize with 1 integrin, and MTCBP-1 can bind to the cytoplasmic domain of MT1-MMP, suppressing its pro migratory effects.

Part of the phenotype could be due to excess MT1-MMP or MMP-9, but RECK may also have other important biological roles besides MMP inhibition. Chondroitin/heparan sulfate proteoglycans, Testican 1 and 3 and a splicing variant of Testican 3, N-Tes, have also been shown to inhibit MT1-MMP (Nakada et al., 2001), although the mechanisms of inhibition are not known.

1.8.3.4 Regulation of MMP-2 activation by MT1-MMP

Since proMMP-2 activation is an important step for cancer cells to invade into basal lamina, the mechanism of activation has thus been extensively studied. This process is not based on a simple interaction of proMMP-2 and MT1-MMP but directly involves, as a third player, its endogenous inhibitor TIMP-2 (Strongin et al., 1995). The catalytic domain of MT1-MMP expressed on the cell surface forms a complex with the N-terminal inhibitory domain of TIMP-2 to form an enzyme-inhibitor complex (Fig. 1.12).

The exposed C-terminal domain of TIMP-2 has a high affinity for the hemopexin domain of proMMP-2, leading to the formation of an MT1-MMP/TIMP-2/proMMP-2 ternary complex (Strongin et al., 1995). Formation of this complex is absolutely essential and proMMP-2 activation can not occur without TIMP-2 (Butler et al., 1998; Kinoshita et al., 1998; Wang and Keiser, 2000). Since MT1-MMP in this complex is inhibited by TIMP-2, another MT1-MMP free from TIMP-2 is required to carry out the activation of proMMP-2. To promote molecular interaction between a free MT1-MMP molecule and the ternary complex of MT1-MMP/TIMP-2/proMMP-2, MT1-MMP forms a homo-oligomer complex through its hemopexin domains and/or transmembrane/cytoplasmic domains (Itoh et al., 2001; Lehti et al., 2002). Within this complex, one MT1-MMP acts as a receptor and the other as an activator, forming a proMMP-2 activation complex. Formation of this homo-oligomer complex is important in the activation process on the cell surface: in fact, when a catalytic domain deletion mutant of MT1-MMP is overexpressed,

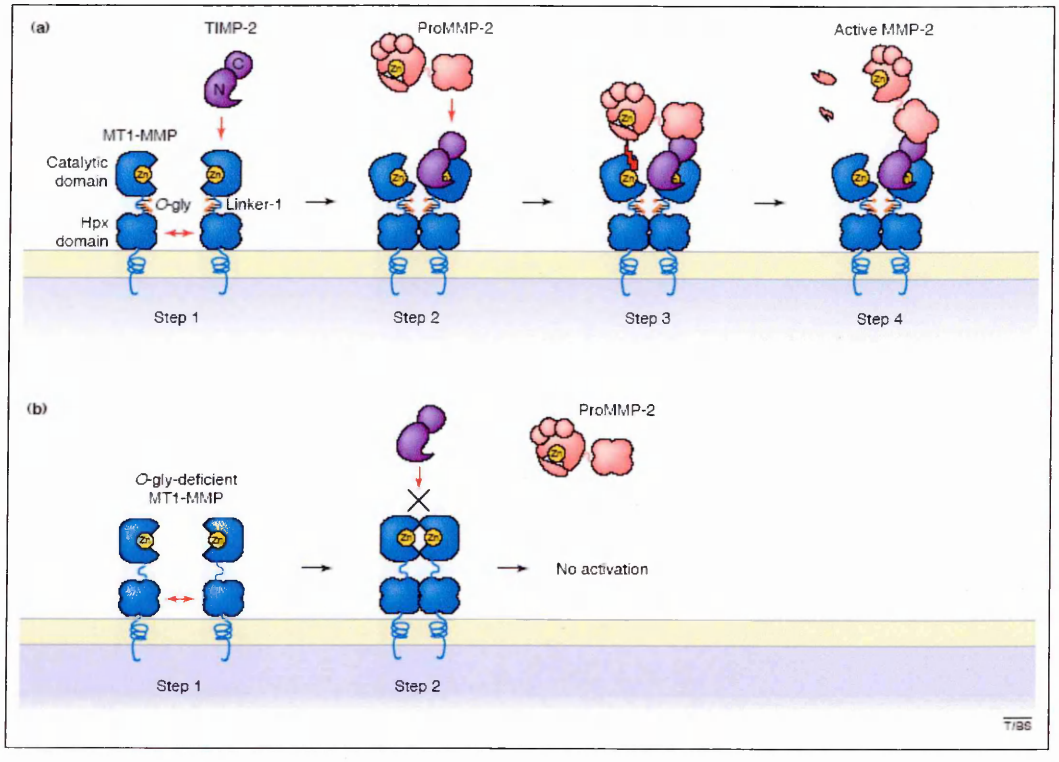


Fig. 1.12. Current model of proMMP-2 activation by MT1-MMP (reproduced from Itoh and Seiki, 2004). (a) In the current model, MT1-MMP (blue) forms a homodimer complex through the hemopexin (Hpx) domains (Step 1). One of the enzymes binds its endogenous inhibitor – tissue inhibitor of metalloproteinase 2 (TIMP-2; purple) – through the catalytic site of the enzyme and the inhibitory site of TIMP-2 (Step 2). The Hpx domain of proMMP-2 (red) has an affinity for the exposed C-terminal domain of TIMP-2 in the complex, resulting in formation of a (MT1-MMP)2–TIMP-2–proMMP-2 complex, which places proMMP-2 in an optimal position for activation by TIMP-2-free MT1-MMP in the complex (Steps 3 and 4). MT1-MMP is *O*-glycosylated at linker-1 region. When the enzyme forms a homodimer complex (Step 1), the sugar moiety might sterically enable one of the catalytic domains of MT1-MMP to be accessible for TIMP-2 binding (Step 2). It is also possible that the sugar inhibits the interaction of a molecule, which inhibits the binding of the enzyme to TIMP-2. This permits further interaction of proMMP-2 with the TIMP-2 (Step 3) and subsequent activation of proMMP-2 (Step 4). The proteolytic action of MT1-MMP on proMMP-2 is represented by a red lightening bolt. **(b)** Glycosylation-defective mutant MT1-MMP enzymes form homophilic complexes (Step1), but the steric arrangement of catalytic domains does not allow TIMP-2 to bind (Step 2). Alternatively, unglycosylated MT1-MMP might interact with a molecule that sterically inhibits TIMP-2 binding to the enzyme. As a result, the mutant enzyme cannot activate proMMP-2.

proMMP-2 activation is effectively inhibited (Itoh et al., 2001). Also, replacement of the hemopexin domain with one derived from another MMP, such as MT4-MMP (Itoh et al., 2001) or MMP-2 (Cao et al., 2004) significantly inhibits activation, presumably because it blocks formation of the homo-oligomer complex.

The trimolecular complex facilitates the first cleavage of proMMP-2 prodomain by a neighboring TIMP-2-free active MT1-MMP, thereby generating a 64 kDa intermediate MMP-2 form. Full activation of the intermediate MMP-2 species is achieved by an intermolecular autocatalytic process (Atkinson et al., 1995; Murphy et al., 1999) or by the action of the plaminogen/plasmin system (Baramova et al., 1997; Sounni et al., 2002). This activation is completely inhibited at high concentrations of TIMP-2, TIMP-3, TIMP-4, but not TIMP-1 (Butler et al., 1999; Lafleur et al., 2001). Therefore, proMMP-2 activation occurs in a TIMP-2-dependent two-step cleavage mechanism. TIMP-2 can be substituted by TIMP-3 in this process, but not by TIMP-1 (Lafleur et al., 2001). TIMP-4, however, which is considered a better MT1-MMP inhibitor, is not able to substitute for TIMP-2 in proMMP-2 activation (Toth et al., 2000). Whereas TIMP-2 acts as a positive regulator of MT1-MMP activity at low concentrations, it blocks proMMP-2 activation at higher concentrations (Hernandez-Barrantes et al., 2000; Murphy et al., 2003; Worley et al., 2003).

The formation of this MT1-MMP/TIMP-2/proMMP-2 trimolecular complex is post-translationally regulated by the O-glycosylation pattern of MT1-MMP and is abolished in cells transfected with MT1-MMP mutants and/or treated with inhibitors of O-glycolysation; the O-glycosylation of MT1-MMP is associated with the recruitment of TIMP-2 to the cell surface (Wu et al., 2004) (for more details, see also Fig. 1.12).

Interestingly, homo-oligomer complex formation can be also upregulated by the expression of a constitutively active form of Rac1, a small GTPase which generates extensive lamellipodia (Itoh et al., 2001). Under these conditions, MT1-

MMP exclusively localizes at the lamellipodia edge, and this is accompanied by enhanced proMMP-2 activation (Itoh et al., 2001).

This suggests that homo-oligomer complex formation is a regulated process and may be one of the mechanisms that determines the functional activity of MT1-MMP. In cell-free systems, proMMP-2 can be activated by the recombinant catalytic domain of MT1-MMP (Will et al., 1996), a domain which does not form a homo-oligomer complex. It is plausible that homo-oligomer complex formation is needed to facilitate the activation process on the cell surface, and the ancillary domains, including hemopexin, transmembrane, and cytoplasmic domains, play a role in this event. It is also possible that other proteins, which interact with MT1-MMP mediate clustering of the enzyme, as suggested by a report showing that a hemopexin domain-deleted mutant of MT1-MMP activates proMMP-2 (Wang et al., 2004a). This may be attributed to transmembrane/cytoplasmic domain-mediated homo-oligomer complex formation (Lehti et al., 2002). It may be important to evaluate the contribution of each domain to homo-oligomer formation for proMMP-2 activation.

1.8.3.5 Cell surface localization of MT1-MMP

When cells migrate in tissue, degradation of ECM barrier is essential, but only in the direction of migration, because ECM is also important for mechanical support. To achieve such focal degradation, cells localize their MT1-MMP at lamellipodia, the migration front of the cells (Itoh et al., 2001; Mori et al., 2002; Sato et al., 1997). This localization is achieved by interaction of MT1-MMP with CD44 through the hemopexin domain of the enzyme and stem region of CD44 (Mori et al., 2002). CD44 in turn is associated with F-actin through its cytoplasmic domain by interacting with Ezrin/Radixin/Moesin proteins. This associates MT1-MMP indirectly with F-actin. Overexpression of the cytoplasmic domain deletion mutant of CD44 abrogated MT1-MMP association with F-actin presumably by competing for the interaction of MT1-MMP with full-length CD44

(Mori et al., 2002). This completely abolishes localization of MT1-MMP to the lamellipodia (Mori et al., 2002), suggesting that association with F-actin is critical for localization of both CD44 and MT1-MMP to the cell front. The interaction of MT1-MMP with CD44 through the hemopexin domain of the molecule is also required for CD44 shedding by the enzyme (Suenaga et al., 2005).

It has been also shown that MT1-MMP can be co-localized with β1 integrin (Ellerbroek et al., 2001; Wolf et al., 2003). It is not clear whether this is due to a direct/indirect interaction, but these reports suggest that MT1-MMP and β1 integrin are functioning in the same area on the cell surface.

1.9 Trafficking and intracellular regulation of MT1-MMP

MT1-MMP has been shown to be internalized by clathrin-dependent and caveolae-dependent pathways (Jiang et al., 2001; Remacle et al., 2003; Uekita et al., 2001) (Fig. 1.13). Because internalization removes MT1-MMP from the cell surface, this can be a mechanism of downregulation. However, almost paradoxically, the internalization process appears to be essential for the enzyme to promote cell migration (Uekita et al., 2001). Clathrin-dependent internalization of MT1-MMP is mediated through the C-terminal cytoplasmic tail. The μ2 subunit of adaptor protein 2 (AP-2), which mediates incorporation of membrane proteins into clathrin cages, interacts with the LLY_{573} sequence in the cytoplasmic tail of MT1-MMP (Uekita et al., 2001).

When LLY_{573} was mutated to alanines or deleted, the internalization of MT1-MMP was inhibited, and the mutant MT1-MMPs could not promote cell migration (Uekita et al., 2001). MT1-MMP is also internalized through caveolae (Remacle et al., 2003). Structural or sequence requirements for this type of internalization are not known, but it is generally thought that localization to the membrane lipid rafts is essential since caveolae are lipid rafts containing caveolin

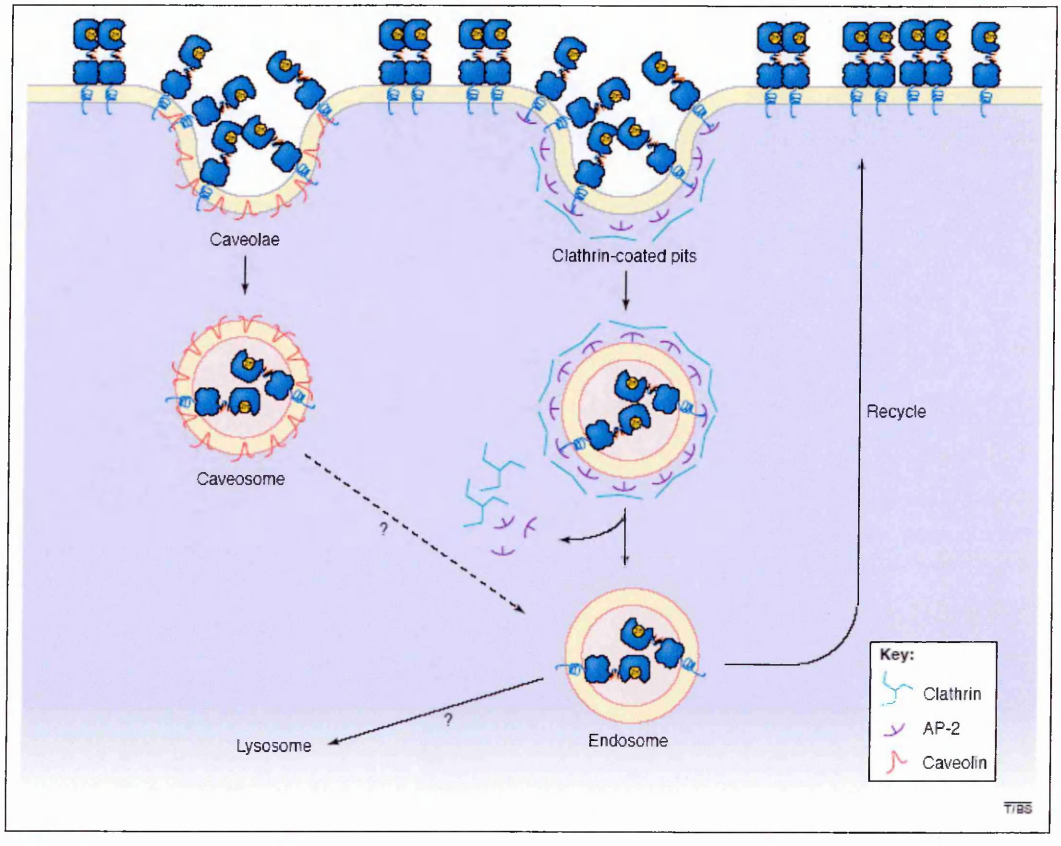


Fig. 1.13. Internalization and recycling pathways of MT1-MMP (reproduced from Itoh and Seiki, 2004). MT1-MMP (blue) is internalized via the clathrin-mediated pathway. The cytoplasmic domain of MT1-MMP contains the motif of Leu-Leu-Tyr573, which binds to the 2 subunit of adaptor protein 2 (AP2; purple), facilitating a formation of clathrin-coated pit. The cytoplasmic domain of MT1-MMP also contains the Asp-Lys-Val582 motif, which is essential for the enzyme to be recycled to the cell surface through endosomes. MT1-MMP is also internalized via caveolae-mediated pathway, but the fate of MT1-MMP in the caveosome is not clear.

(Varma and Mayor, 1998). Galvez *et al.* have demonstrated that caveolae-mediated internalization of MT1-MMP also plays an important role in MT1-MMP-mediated endothelial cell migration on a collagen substratum (Galvez et al., 2004).

A part of internalized MT1-MMP was shown to be transported to the CD63 positive lysosomes for degradation (Takino et al., 2003). Interestingly, CD63 tetraspanin, a well established component of late endosomal and lysosomal membranes, interacts with MT1-MMP directly through the N-terminus of CD63 and the hemopexin domain of MT1-MMP (Takino et al., 2003). Expression of CD63 accelerates internalization and lysosomal degradation of MT1-MMP, and it requires both the hemopexin and the cytoplasmic domains of MT1-MMP (Takino et al., 2003). Therefore, CD63 is one of the regulators of MT1-MMP trafficking.

MT1-MMP is also recycled back to the cell surface after internalization. Wang *et al.* have shown that the DKV_{583} motif in the C-terminus of the cytoplasmic domain is a critical sequence (Wang et al., 2004b). Deletion of DKV_{583} inhibits recycling of MT1-MMP. A similar motif, EWV is present at the C-terminus of MT2-, MT3- and MT5-MMP, and MT3-MMP is shown to be able to co-recycle with MT1-MMP in MDCK cells (Wang et al., 2004b). Mint-3 has been identified as a protein that interacts with the EWV motif of MT5-MMP (Wang et al., 2004b). Mint-3 upregulates surface levels of MT5-MMP, presumably by increasing the recycling frequency. It would be interesting to know if Mint-3 can also regulate trafficking of MT1-MMP.

Another protein which interacts with the cytoplasmic domain of MT1-MMP has been named MT1-MMP cytoplasmic-binding protein-1 (MTCBP-1) (Uekita et al., 2004). MTCBP-1 does not affect proMMP-2 activation by MT1-MMP but suppresses MT1-MMP-mediated cell migration. Interestingly many cancer cell lines, which express high levels of MT1-MMP express low level of MTCBP-1 and normal fibroblasts which express low levels of MT1-MMP express a high levels of MTCBP-1 (Uekita et al., 2004). This inverse correlation suggests

that MTCBP-1 may act as an invasion suppressor.

1.10 Main findings of this thesis

In order to understand the role of intracellular trafficking, membrane sorting and cell surface release of matrix metalloproteinases in tumor invasion and metastatic dissemination, as the main topic of my PhD project, I decided to focus my attention mainly on MT-MMPs since they represent a restricted subset of MMPs, which may also cleave cell surface proteins and serve as receptors and activators for secreted MMPs. In addition, the severe connective tissue disorders of MT1-MMP knockout mice, and the efficacy of MT1-MMP to promote, both directly and indirectly, migratory and invasive phenotype of normal and neoplastic cells, prompted me not only to characterize the molecular mechanisms of MT1-MMP activation but also to define its subcellular activation compartment.

In chapter 3 I report that MT1-MMP is mainly associated with detergent-resistant membranes and that MT1-MMP undergoes proteolytic processing only if associated with detergent-soluble membranes. In chapter 4 I report that MT1-MMP activation occurs intracellularly in a post-Golgi, pre-plasma membrane compartment. In chapter 5 I discuss the biological meaning of these data in the regulation of MT1-MMP-dependent activities.

CHAPTER 2

Materials and Experimental Procedures

2.1 General Materials

Sodium dodecyl sulphate (SDS), glycine, DL-dithiothreitol (DTT), potassium acetate, TRIZMA base, magnesium acetate, Tris[Hydroxymethyl]aminomethane (Tris), ethidium bromide, ethylene glycol-bis (2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), ethyl-enediaminetetraacetic acid (EDTA), KH_2PO4, Na_2HPO4, NaH_2PO4, $MgSO_4$, bovine serum albumine (BSA), aprotinin, benzamidine, leupeptin, pepstatin, 1,10-phenanthroline, phenylmethylsulphonyl fluoride (PMSF), brefeldin A (BFA), kanamycin, gelatin, methylcellulose, uranyl acetate, sucrose, tunicamycin, lysozyme, sodium azide, sodium deoxycholate, tannic acid, methyl-β-cyclodextrin, lovastatin, mevalonate, rhodamine and fluorescein 5-isothiocyanate (FITC) were from Sigma-Aldrich (WI, USA). NaCl, $Na_2B_4O_7$, HCl, NaOH, KOH, NH_4Cl, $(NH_4)SO_4$, glacial acetic acid, ethanol, trichloroacetic acid (TCA) were from Carlo Erba (Italy). Piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES), 4-(2-Hydroxy-ethyl)-piperazine-1-ethane-sulfonic acid (HEPES), glycerol, KCl, $MgCl_2$, and $CaCl_2$ were from Merck (Germany). Triton X-100 and Nonidet P-40 (NP-40) were from Fluka (Sigma-Aldrich, WI, USA). The broad-range MMP inhibitor BB-94 was from British Biotechnology (UK). Other materials will be specified for each procedure.

2.2 Cell culture and transfection

2.2.1 Materials

A375MM cells (hereafter referred to as A375 cells), an established human melanoma cell line (Kozlowski et al., 1984), were kindly provided by Dr. G. Egea (University of Barcelona, Spain). Human fibrosarcoma HT1080 cells were purchased from American Tissue Type Collection (VA, USA). Modified Eagles Medium (MEM), Dulbecco's Modified Eagles Medium (DMEM), F12 Medium, Fetal Calf Serum (FCS), non essential amino acids (NEA), penicillin, streptomycin, trypsin/EDTA, and L-glutamine were from Gibco (NY, USA). All the plastic materials were from Corning (NY, USA). Filters (0.45 and 0.2 μm) were from Amicon (MA, USA).

2.2.2 Growth media

A375 cells were maintained in DMEM/F12 (1:1) nutrient mixture supplemented with 10% FCS, 4 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin. HT1080 cells were grown in MEM supplemented with 10% FCS, 4 mM L-glutamine, 0.1 mM NEA, 100 U/ml penicillin and 100 μg/ml streptomycin.

2.2.3 Growth conditions

All cell lines were grown under a controlled atmosphere in the presence of 5% CO2 at 37°C. Cells were grown in a flask until 80-100% confluence. The medium was removed and trypsin/EDTA solution (0.05% trypsin, 0.02% EDTA) added for 2-5 minutes. The medium was then added back to block protease action and the cells were collected in a plastic tube. After centrifugation for 5 min at 300 x g, the pellet was resuspended in fresh medium.

2.2.4 Cell transfection

For transfection, cells were plated at 50% confluence, and the next day were incubated 1 hour at 37°C with 0.45 $\mu g/cm^2$ DNA of interest and 2 $\mu l/\mu g$ DNA TransFast (Promega, WI, USA). Complete medium was then added. Experiments involving overexpression of exogenous proteins were usually performed the day after transfection.

2.3 cDNA Constructs

MT1-MMP cDNA encoding an open reading frame from amino acid residues Met^{1}-Val^{583}, GFP-tagged-MT1-MMP and the catalitically inactive MT1-MMP mutant (E240A) were cloned in a pcDNA3.0 expression vector (Gibco, NY, USA), (as described in Cao et al., 1996), and kindly provided by Dr. S. Zucker (Stony Brook University, NY, USA). The FLAG-tagged-MT1-MMP (F112) cDNA was cloned in a pcDNA3.0 expression vector (Gibco, NY, USA), (as described in Wu et al., 2004), and kindly provided by Dr. M.S. Stack (Northwestern University, Chicago, IL, USA). The FLAG-tagged α1-PDX cDNA, (described in Jean et al., 1998), was kindly provided by Dr. G. Thomas (Oregon Health Sciences University, Portland, OR, USA) and subcloned in pcDNA3.0, as described below. GFP-tagged furin cDNA was kindly provided by Dr. J. Bonifacino (NIH, Bethesda, MD, USA). GFP-tagged Rab4, Rab5, Rab7, Rab11 were kindly provided by Dr. M. Zerial (Max-Planck, Dresden, Germany). The ts045 temperature-sensitive Vesicular Stomatitis Virus G glycoprotein mutant (VSV-G)-GFP chimera was a generous gift of Dr. J. Lippincott-Schwartz (NIH, Bethesda, MD, USA).

2.4 General subcloning procedures

2.4.1 Materials

Restriction enzymes were from Amersham Pharmacia Biotech (NJ, USA). T4 DNA ligase, DNA molecular size standards were from Gibco (NY, USA). "QIAprep Spin Miniprep Kit" and "Qiagen Plasmid Maxi Kit" were from Qiagen (CA, USA). Tryptone Peptone, Yeast extract and agar were from Difco (Becton Dickinson, MD, USA). 3-Morpholino-propane-sulfonic acid (MOPS), RbCl and $MnCl_2$ were from Sigma Aldrich (WI, USA).

2.4.2 Solutions and media

Luria Broth (LB): 1% (w/v) Tryptone Peptone, 0.5% (w/v) Yeast extract, 1% (w/v) NaCl; autoclaved 15 min at 121°C. LB-agar: LB plus 1.5% (w/v) agar: autoclaved 15 min at 121°C. Tfb1: 30 mM potassium acetate, 100 mM RbCl, 10 mM $CaCl_2$, 50 mM $MnCl_2$, 15% (v/v) glycerol pH 5.8. Tfb2: 10 mM MOPS, 75 mM $CaCl_2$, 10 mM RbCl, 15% (v/v) glycerol pH 7.0. TE buffer: 10 mM Tris-HCl pH 8.0, 1 mM EDTA. 50X TAE buffer (1 litre): 242 gr. TRIZMA base, 57.1 ml glacial acetic acid, 100 ml 0.5 M EDTA.

2.4.3 DNA agarose gel

Agarose gels were prepared by dissolving agarose (Bio-Rad Laboratories, UK) in 1X TAE buffer and heating with a microwave oven. 0.5 μg/ml of ethidium bromide were added and the gel was poured and run on an agarose gel apparatus from Bio-Rad Laboratories (UK). 0.3-0.5 μg of DNA standards were loaded and often used as a reference to give an approximate estimation of the amounts of DNA samples.

2.4.4 PCR amplification of DNA inserts

To amplify specific regions of DNA inserts, PCR was performed incubating 10 ng DNA-plasmid as template in 50 μl of 20 mM Tris-HCl pH 8.8, 10 mM KCl, 2 mM $MgSO_4$, 10 mM $(NH_4)SO_4$, 0.1% Triton X-100, 0.1 mg/ml nuclease free BSA, 1 μM each oligonucleotide, 200 μM each dNTP, 2.5 U Pfu Turbo DNA Polymerase. All reagents, except DNA and oligonucleotides, were from Stratagene (CA, USA). Oligonucleotides were purchased from Gibco (NY, USA) or from Sigma-Aldrich (WI, USA). The PCR reaction mixtures were subjected to 35 temperature cycles in a programmable thermal cycler (PerkinElmer, MA, USA). Melting-, annealing-, and elongation-temperatures were adjusted according to the features of template and primers. To facilitate the subsequent subcloning of PCR products, the forward and the reverse primers were provided with restriction sites at their 5' ends (see specific sections).

2.4.5 Restriction and ligation

DNA (vectors and inserts) were cut with 5 U/μg of appropriate restriction enzymes in the buffer supplied with each enzyme by Amersham Pharmacia Biotech (NJ, USA). After restriction, enzymes were usually inactivated by incubating at 65-75°C for 10-20 min, according to the manufacturer's instructions, and loaded on 1% agarose gel. The bands of interest were cut out of the gel with a sterile scalpel, and DNA was extracted from the gel with Qiaex II extraction kit (Qiagen, CA, USA), according to the manufacturer's instructions. DNA was eluted in 10 mM Tris-HCl pH 8.0. To ligate vector and insert, about 100 ng of vector and about 3-fold molar amounts of insert were incubated with 1U T4 DNA ligase in T4-DNA-ligase buffer (Gibco, NY, USA) 10 min at room temparature (RT).

2.4.6 Preparation of competent bacteria

A single colony of XL-1Blue *E. Coli* bacteria (Stratagene, CA, USA) was picked from a LB-agar plate and used to inoculate 10 ml of LB. Bacteria were grown O.N., the culture was diluted in 190 ml of fresh LB and incubated at 37 °C until the optical density reached 0.5 (at 600 nm). Bacteria were harvested by centrifugation at 4,000 x g for 10 min at 4 °C. The bacterial pellet was resuspended in 64 ml of Tfb1 and left on ice for 1 to 2 hours. After centrifugation and resuspension of the pellet in 8 ml of Tfb2 cells were frozen in liquid nitrogen and stored at –80°C in 400 μl aliquots.

2.4.7 Transformation of bacteria

The DNA plasmid of interest (10 ng of uncut plasmid or half of a ligation reaction) was added to 200 μl of competent bacteria (see previous section) previously thawed on ice. After gentle mixing, the cells were left on ice for 30 min, heat shocked for 45 sec at 42°C, and after addition of 800 μl LB, incubated under shaking (200 rpm) at 37°C for 45 min. Bacteria were plated on LB agar containing the appropriate selective antibiotic and incubated O.N., at 37 °C. The next day, an isolated bacterial colony was picked and used to inoculate 2ml of LB containing the appropriate antibiotic. The culture was incubated at 37 °C O.N. 300 μl of 50% (v/v) sterile glycerol were added to 700 μl of the bacterial culture and stored at -80 °C.

2.4.8 Small-scale preparations of plasmid DNA (miniprep)

The clones obtained after transformation of ligation reaction were usually screened by miniprep and subsequent restriction analysis. Isolated bacterial colonies were picked and inoculated in 5 ml of LB containing the appropriate antibiotic. After O.N. growing at 37°C under continuous shacking (200 rpm), 700 μl of the culture was mixed with 300 μl 50% (v/v) sterile glycerol and stored at

–80°C, while the rest of the culture was chilled on ice and centrifuged 10 min at 4,000 x g. The DNA was extracted using the "QIAprep Spin Miniprep Kit" (Qiagen, CA, USA), according to the manufacturer's instructions and analysed by restriction analysis and separation on agarose gel.

2.4.9 Large-scale preparation of plasmid DNA (maxiprep)

A small amount of bacteria transformed with the plasmid of interest, were scraped from the glycerol stock, inoculated in 2 ml of LB containing the appropriate selective antibiotic and grown at 37°C under continuous shacking (200 rpm) for 6-8 hours. This pre-culture was used to inoculate 200 ml of LB containing selective antibiotic. After an O.N. incubation, bacteria were collected by centrifugation at 6000 x g in a JA14 rotor for 10 min at 4 °C and processed according to the maxi-plasmid purification protocol of the "Qiagen Plasmid Maxi Kit". The DNA obtained was resuspended in TE buffer and stored at -20 °C.

2.5 General biochemical procedures

All the experiments involving the following procedures were performed at least three times, in duplicate.

2.5.1 Materials

Ponceau red, bromophenol blue, coomassie brilliant blue R250, ammonium persulphate (APS), N,N,N',N'-tetramethylethylenediamine (TEMED) were from Sigma-Aldrich (WI, USA). β-mercaptoethanol was from Fluka (Sigma-Aldrich, WI, USA). Methanol, butanol, and glacial acetic acid were from Carlo Erba (Italy). Secondary antibodies conjugated with Horse-Radish Peroxidase (HRP) and directed against mouse or rabbit IgGs were from Calbiochem (CA, USA).

2.5.2 Solutions

Acrylamide stock solution: 40% (w/v) acrylamide-bisacrylamide (37.5:1) (Eurobio, France). Running buffer: 25 mM TRIZMA base, 0.2 M glycine, 0.1% (w/v) SDS. SDS-sample buffer: 62.5 mM Tris-HCl pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol and 0.1% (w/v) bromophenol blue. Transfer buffer: 25 mM TRIZMA base, 0.2 M glycine, 20% (v/v) methanol. Phosphate buffered saline (PBS): 1.5 mM KH_2PO4, 8 mM Na_2HPO4, 2.7 mM KCl, 137 mM NaCl. T-PBS: 0.05% (w/v) Triton X-100 in T-PBS. Blocking solution: 1% (w/v) BSA in T-PBS.

2.5.3 Sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE)

2.5.3.1 Assembly of polyacrylamide gels

Two 16 x 18 cm plates were used for assembling a regular gel, while 32 x 18 glasses were used for assembling longer gels. The plates were assembled to form a chamber using two 1.5 mm plastic spacers aligned on the lateral edges of the plates. The plates were then fixed using two clamps and mounted on a plastic base which sealed the bottom. All equipment was from Hoefer Scientific Instruments (Germany). The 'running' polyacrylamide gel was prepared by mixing H_2O, 40% (w/v) acrylamide-bisacrylamide solution, 1.5 M Tris-HCl pH 8.8, 10% (w/v) SDS, in order to have 4% (w/v) acrylamide, 375 mM Tris-HCl, 0.1% (w/v) SDS. Then, 0.06% (w/v) of ammonium persulphate and 0.06% (v/v) TEMED were added, the solution was pipetted and poured into the gap between the plates, leaving about 5 cm for the stacking gel. Immediately after pouring, the gel was covered with a layer of butanol and left at RT for about 1 hour. The butanol layer was removed. The 'stacking' polyacrylamide gel was prepared by

mixing H_2O, 40% (w/v) acrylamidebisacrylamide solution, 0.5 M Tris-HCl pH 6.8, 10% (w/v) SDS, to have 4% (w/v) acrylamide, 125 mM Tris-HCl, 0.1% (w/v) SDS. Then, 0.1% (w/v) ammonium persulphate and 0.07% (v/v) TEMED were added, the solution pipetted and poured on the 'running' gel. Immediately, a 15-well comb was inserted between the glasses and left for1 hour at RT.

2.5.3.2 Sample preparation and run

Samples were prepared by adding SDS-sample buffer, incubating at 100°C for 5-15 min in a Multi-Block Heater (Lab-Line, IL, USA), cooled at RT, briefly centrifuged and loaded on the gel. One or two wells were loaded with 3 μl of Rainbow recombinant protein molecular mass markers (Amersham Pharmacia Biotech, NJ, USA). The gel was then transferred into the electrophoresis apparatus (Hoefer Scientific Instruments, NJ, USA) and the electrophoresis was carried out under a constant current of 8 mA (for O.N. runs) or 40 mA (for short 4-hour runs).

2.5.3.3 Evaluation of protein concentration

When required, protein concentration was evaluated using a commercially available protein assay kit (Bio-Rad Laboratories, UK) according to the manufacturer's instructions.

2.5.3.4 TCA-based protein precipitation

10% TCA and 0.4 mg/ml sodium deoxycholate were added from a 5X TCA/deoxycholate stock to 500 μl of the third elution fractions. The mixture was vortexed and incubated on ice for 1 hour. After a 10 min centrifugation at 13,000 x g at 4°C, the supernatant was collected and the pellet was washed twice, by adding cold acetone, centrifuging as indicated above and removing the supernatant. At the end, the pellet was dried under gentle aspiration, resuspended

in SDS-sample buffer, incubated at 100°C for 15 min vortexing every 5 min and subjected to SDS-PAGE.

2.5.3.5 Coomassie brilliant blue Staining

Gels were incubated in staining solution (methanol, H_2O, acetic acid 5:5:2, and 0.1 % coomassie brilliant blue) for 2 hours, washed and then destained with 30% methanol 10 % acetic acid.

2.5.3.6 Gel drying

When required, the gels were washed with H_2O and 10% polyacrylamide, briefly incubated with 4% (v/v) glycerol and dried with a gel dryer (Hoefer Scientific Instruments, Germany) at 80°C under vacuum for 4 hours.

2.5.4 Western Blotting

2.5.4.1 Protein transfer onto nitrocellulose

The polyacrylamide gel was soaked for 15 min in transfer buffer, placed on a sheet of 3MM paper (Whatman, NJ, USA) and covered by a nitrocellulose filter (Schleicher & Schuell, Germany). The filter was covered by a second sheet of 3MM paper, to form a "sandwich" which was subsequently assembled into the blotting apparatus (Hoefer Scientific Instruments, NJ, USA). Protein transfer occurred at 500 mA for 4 hours or at 125 mA O.N. At the end of the run, the sandwich was disassembled and the nitrocellulose filter was soaked in 0.2% (w/v) ponceau red and 5% (v/v) acetic acid for 5 min to visualize the protein bands, and then rinsed with 5% acetic acid to remove the excess of unbound dye.

2.5.4.2 Probing nitrocellulose with specific antibodies

The nitrocellulose filters were cut into strips with a razor blade. Strips containing the proteins of interest were incubated in T-PBS containing 5% skim milk powder (Fluka, Sigma-Aldrich, WI, USA) for 1 hour at RT, and then with the primary antibody at the appropriate working concentration in blocking solution (see Table 2.1 for the working diluitions of antibodies used in this thesis). After 2-3 hour incubation at RT, or O.N. incubation at 4°C, the antibody was removed and the strips washed in T-PBS twice for 10 min. The strips were next incubated for 1 hour with the appropriate HRP-conjugated secondary antibody diluted 1:2000 in blocking solution and washed twice in T-PBS for 10 min and once in PBS for 3 min. After washing, the strips were incubated with ECL reagents from Amersham Pharmacia Biotech (NJ, USA), according to the manufacturer's instructions, for electrochemiluminescence-based detection. The strips were incubated with the ECL developing solution for 1 min at RT and directly exposed to BioMax Films (Kodak, NY, USA).

Table 2.1. List of antibodies used in Western blot experiments

Specificity (antibody name)	Owner (Company or Group's Head)	Animal source	Dilution
MT1-MMP (MMR2)	Our laboratory	Rabbit	1:10,000
MT1-MMP (M3927)	Sigma-Aldrich (WI, USA)	Rabbit	1:1,000
α1-Antitrypsin (A0012)	DAKO A/S (Denmark)	Rabbit	1:500
Caveolin-1 (SC-894)	Santa Cruz (CA, USA)	Rabbit	1:1,000
FLAG (F7425)	Sigma-Aldrich (WI, USA)	Rabbit	1:200

FLAG (M1) (F3040)	Sigma-Aldrich (WI, USA)	Mouse	1:100
FLAG (M2) (F3165)	Sigma-Aldrich (WI, USA)	Mouse	1:100

2.6 Preparation of antibodies

2.6.1 Materials

Glutathione, lysozyme, isopropyl-β-D-1-thiogalactopyranoside (IPTG), sodium azide, Freund's adjuvant, borate, ethanolamine and triethanolamine were from Sigma-Aldrich (WI, USA). The glutathione-Sepharose resin was from Amersham Pharmacia (Italy).

2.6.2 Generation of MT1-MMP antibodies

In all experiments and unless indicated otherwise, MT1-MMP was immunodetected or immunoprecipitated with rabbit polyclonal antisera (MMRs) raised against recombinant GST-fused polypeptides corresponding to: a) the MT1-MMP propeptide domain (amino acids 34-112) (for MMR3 antibody) and b) the propeptide-deleted extracellular portion of MT1-MMP (amino acids 113-538) (for MMR1 and MMR2 antibodies).

The cDNA coding for the MT1-MMP propeptide domain was amplified by PCR (see above) with the following primers: 5'-CCGGAATTCCCCGAAGCCTGGCTACAGCAA-3' (forward primer), and 5'-GAGTCGACCTAGCGCTTCCTTCGAACATTG-3' (reverse primer). The PCR reaction mixture was subjected to 35 temperature cycles (30 sec melting at 95°C, 30 sec annealing at 55°C and 1 min elongation at 72°C). The PCR product was digested with EcoRI and SalI and subcloned in EcoRI/SalI-digested pGEX-4T1

(Amersham Pharmacia Biotech, NJ, USA; see also section for general subcloning procedures) generating the pGEX-4T1+(MT1-MMP)propeptide plasmid. Glycerol stocks were established and stored at –80°C. The cDNA coding for the propeptide-deleted extracellular portion of MT1-MMP was kindly provided by Dr. S. Zucker (Stony Brook University, NY, USA).

These cDNAs were used to transform competent BL21(DE3) *E. Coli* strain (Stratagene, CA, USA). These bacteria were grown in LB plus kanamycin (50 mg/ml) until the optical density (600 nm) was about 0.7-0.9. At this point, the fusion protein was induced for 3 h at 37°C with 1 mM IPTG. The cells were harvested by centrifugation (6000 x g, JA 10 rotor) for 10 min at 4°C and resuspended in 25 ml of 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, 1 mg/ml lysozyme in the presence of a mixture of protease inhibitors (2.0 μg/ml aprotinin, 0.5 μg/ml leupeptin, 2 μM pepstatin, 0.5 mM 1,10-phenantroline, 1 mM PMSF). 1% Triton X-100 was added, the suspension was stirred for 30 min, and then sonicated for 2 min on ice. The lysed cells were centrifuged for 20 min (1,800 rpm, JA 20 rotor). 2.5 ml of a glutathione-Sepharose resin (Amersham Pharmacia, Italy) was diluted up to 40 ml in PBS to remove the preservative and centrifuged (2,000 x g) for 5 min. The supernatant was discarded and the resin was resuspended again in PBS. This procedure was repeated twice. Supernatant from bacterial lysates were added to the glutathione-Sepharose resin and incubated for 30 min at 4°C with occasional agitation. The beads were washed with 3 x 50 ml PBS, and assembled into a column. Protein elution was started by the addition of 1 ml of 100 mM Tris-HCl, 20 mM glutathione, 5 mM DTT. 1 ml was collected and termed fraction 1. This procedure was repeated six times. Each step of the protein purification was analysed by SDS-PAGE (Fig. 2.1).

The remaining part of this preparation was performed with the help of Mr. G. Di Tullio (Protein analysis facility, Department of Cell Biology and Oncology, Consorzio Mario Negri Sud).

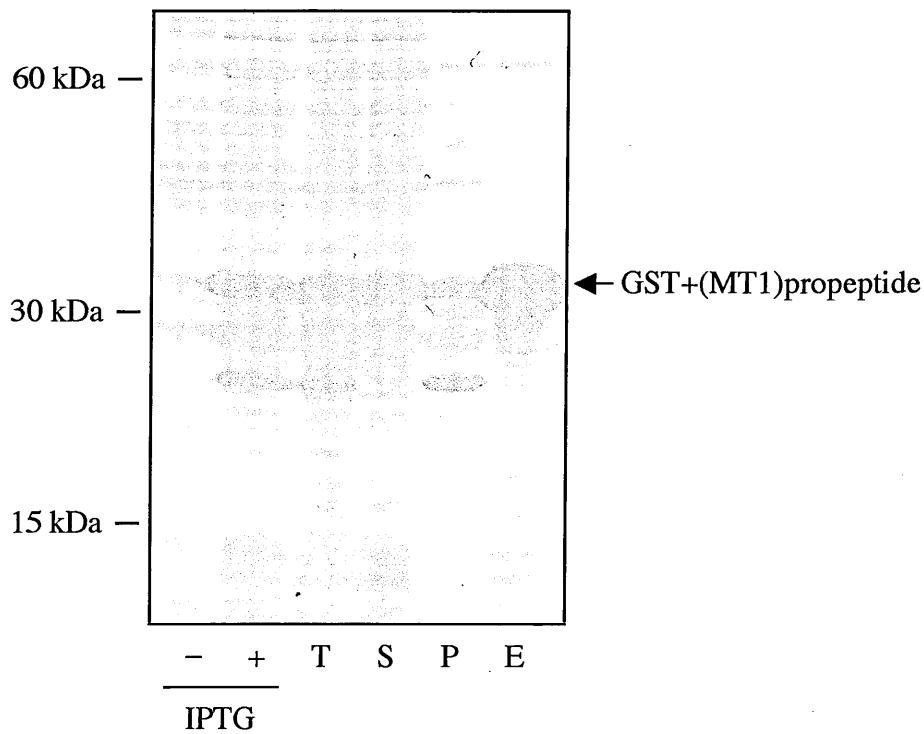


Fig. 2.1. Purification of GST+(MT1)propeptide. The cDNA coding for the MT1-MMP propeptide (amino acids 34-112) was amplified by PCR, as described in materials and methods section, and cloned in the pGEX-4T1 vector to be expressed as a GST-fused chimera from BL21(DE3) *E.Coli.* The expressed protein was then purified by using a glutathione-Sepharose resin. Small amounts of the recombinant protein during different purification steps were analysed by 12% SDS-PAGE, transferred to nitrocellulose membrane and visualized by Ponceau red. Fractions: (-) IPTG-unstimulated bacteria; (+) IPTG-stimulated bacteria; (T) total crude lysates; (S) supernatant; (P) pellet and (E) eluted purified protein. The position of molecular weight standards are indicated on the left.

Purified fusion protein (1mg) was mixed with an equal volume of complete Freund's adjuvant (Sigma Aldrich, WI, USA) and used to immunise New Zealand rabbits. The rabbits were boosted after 15 and 30 days with 500 μg of antigen in incomplete Freund's adjuvant. The rabbits were bled and the presence of anti-MT1-MMP antibody tested by Western blotting.

2.6.3 Affinity purification of MMR2 antiserum

This purification was performed by Mr. G. Di Tullio (Protein analysis facility, Department of Cell Biology and Oncology, Consorzio Mario Negri Sud, Italy). The polyclonal anti-MT1-MMP antibody (MMR2) was affinity-purified on an affinity column prepared by coupling purified GST-fused propeptide-deleted extracellular MT1-MMP portion (5 mg) with glutathione-Sepharose beads (2 ml). To cross-link this fusion construct convalently to Sepharose beads, the beads were first washed twice with 10 ml of 0.2 M borate-NaOH, pH 8.6. After centrifugation (500 x g, 2 min at 4°C), recovered beads were incubated with 20 mM dimethyl pimelimidate-HCl (Pierce, IL, USA) in 0.2 M triethanolamine (2 ml). After 30 min of continuous mixing at RT, the beads were centrifuged and the supernatant discarded. The cross-linking reaction was stopped by adding 10 ml 0.2 M ethanolamine-HCl, pH 8.2, for 60 min. The beads were washed twice with 5 ml of 0.1 M glycine-HCl, pH 2.5 to remove non-covalently-linked molecules. The resin, now ready for the affinity purification, was washed twice with PBS and stored with 0.02% sodium azide at 4 °C until use. The crude rabbit serum raised against the GST-fused polypeptide (2 ml) was loaded onto the resin and, after extensive washing with PBS, the antibodies specifically bound to the column were eluted with 0.1 M glycine, pH 2.5 (5 ml). The pH of the eluted fractions (1 ml each) was adjusted to 7.0 with 1 M Tris, pH 10. The pooled eluate fractions, as well as the flow-though fraction, were dialysed 12-15 hours against PBS plus 0.02% sodium azide and concentrated by ultrafiltration (Centriprep 30; Amicon, MA, USA) to the original serum volume. Purified antibodies were stored at -20°C.

2.7 Preparation of detergent-resistant membranes

2.7.1 Solutions

Phosphate buffered saline (PBS): 1.5 mM KH_2PO4, 8 mM Na_2HPO4, 2.7 mM KCl, 137 mM NaCl. TNE buffer: 50 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, pH 8.0. Lysis buffer: 1% (v/v) Triton X-100 in TNE buffer containing a cocktail of protease inhibitors: 2.0 μg/ml aprotinin, 0.5 μg/ml leupeptin, 2 μM pepstatin, 0.5 mM 1,10-phenantroline, 1 mM PMSF. Solubilization buffer: 0.2% (w/v) SDS in Lysis buffer.

2.7.2 Isolation of Triton X-100-insoluble membranes

TX-100-insoluble membranes were prepared as originally described by Brown and Rose (Brown and Rose, 1992) with the following modifications. Cells at confluence in 35 mm dishes were washed twice with ice-cold PBS containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ and then lysed for 20 min on ice in 0.5 ml of TNE buffer (pH 7.4) containing 1% Triton X-100. Lysates were then collected and ultracentrifuged at 120,000 x g in TL100.1 rotor (Beckman, CA, USA) for 60 min at 4°C. Supernatants, representing the soluble material, were collected in 1.5 ml tubes and prepared for SDS-PAGE with 4X SDS-sample buffer. The pellets, representing the insoluble material, were directly resuspended in SDS-sample buffer. Small amounts of each samples were loaded on gel.

2.7.3 Immunoprecipitation of Triton X-100-insoluble membranes

Cells were seeded on 6-well plates and transfected as described above. Twenty-four hours after transfection confluent cells were washed twice with ice-cold PBS and then incubated with 0.5 ml Lysis buffer on a shaker for 60 min at

4°C. Cell lysates were scraped, collected and ultracentrifuged at 120,000 x g in TL100.1 rotor (Beckman, CA, USA) for 60 min at 4°C. Meanwhile, protein A-Sepharose beads (Amersham Pharmacia, Italy) were washed twice with ice-cold PBS and resuspended in TNE buffer at a final concentration of 42 mg/ml, corresponding to a mixture containing 10% (v/v) beads.

After ultracentrifugation, cleared lysates were collected in 1.5 ml tubes and stored at 4°C. When required, the Triton X-100 insoluble material was also suspended in 0.5 ml solubilization buffer, incubated for 60 min at 37°C and then rapidly cooled at 4°C. Lysates were then incubated on a rolling wheel with 5 µl of polyclonal sera or 1 µl of affinity-purified antibodies for 60 min at 4°C. 50 µl of a 10% protein A-Sepharose beads mixture were added to collect immune complexes for 60 min at 4°C. After a quick spin (13,000 x g for 2 min at 4°C), the supernatant was removed by aspiration and the pellet was washed three times in lysis buffer. The last wash was removed as completely as possible, reaching a final volume of approximately 50 µl. For SDS-PAGE analysis, 50 µl of SDS-sample buffer were added. Samples were heated to 90 °C for 10 min and then loaded onto gel. Samples were also stored at –20°C.

2.7.4 Flotation assay of Triton X-100-insoluble membranes

Flotation assay of Triton X-100-insoluble material was performed using previously published protocols (Arreaza et al., 1994) with the following modifications. Cells were grown to confluence in 100 mm dishes and then washed in ice-cold PBS and lysed with 1% Triton X-100 in TNE buffer (50 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, pH 8.0) for 10 minutes on ice. Lysates were scraped from dishes, adjusted with OptiPrep density gradient medium, a 60% (w/v) solution of iodixanol in water (Sigma-Aldrich, Germany), to a final 35%, and then placed at the bottom of a centrifuge tube. A discontinuous Optiprep gradient (5-30% in TNE) was layered on the top of the lysates and the samples were

ultracentrifuged at 120,000 x g for 16 hours in a swinging-bucket rotor (model SW41, Beckman, CA, USA). One-milliliter fractions were harvested from the top of the gradient. MT1-MMP immunoprecipitation was performed, as described above, with 5 μl of MMR2 antiserum after adding Triton X-100 to the samples to reach the final concentration of 1%. Immunoprecipitates were recovered with protein A-Sepharose (Amersham Pharmacia, Italy), washed twice in TNE, resuspended with SDS-sample buffer and separated by SDS-PAGE; proteins were then electro-transferred to nitrocellulose filters and detected by autoradiography with the Fujifilm bioimaging analyzer system BAS 1800 II (Fuji, Japan) and relative software.

2.8 Pulse-chase analysis

Twenty-four hours after transfection, confluent cells were washed twice with DMEM and then labeled for different times with an amino acid mixture for *in vitro* protein radiolabeling containing L-[^{35}S]Methionine and L-[^{35}S]Cysteine (Redivue, Amersham Pharmacia, Italy) at a final concentration of 0.1 mCi/ml, in DMEM lacking L-Methionine and L-Cysteine (Gibco, NY, USA). Depending on the experiment, cells were then chased for different times in complete growth medium. Cells were thus cooled on ice and washed twice with ice-cold PBS. After extraction with 1% Triton X-100 in TNE, soluble and insoluble fractions were separated by ultracentrifugation at 4°C (120,000 x g, 1 hour). The pellet, containing Triton X-100-insoluble material, was further incubated with 0.2% SDS, 1% Triton X-100 in TNE at 37°C for 1 hour. MT1-MMP immunoprecipitation was performed with MMR2 antiserum. Immunoprecipitates were recovered with protein A-Sepharose (Amersham Pharmacia, Italy), washed twice in TNE, resuspended with sample buffer and separated by SDS-PAGE; proteins were then transferred to nitrocellulose filters and detected by autoradiography. Quantitative analysis was performed with the Fuji BAS 1800 II software (Fuji, Japan).

2.9 Biotinylation assay of cell surface proteins

Twenty-four hours after transfection, confluent cells were cooled on ice, washed twice with ice-cold PBS and incubated twice for 20 minutes each with 1 mg/ml NHS-biotin (Pierce, IL, USA) in PBS at 4°C. Next, cells were washed with PBS and excess NHS-biotin was quenched for 20 minutes with 50 mM NH_4Cl in PBS. After extraction with 1% Triton X-100 in TNE buffer (pH 7.4) at 4°C, soluble and insoluble material was separated by ultracentrifugation at 4°C (120,000 x g, 60 min). The pellet, containing Triton X-100-insoluble material, was further incubated with 0.2% (w/v) SDS, 1% (v/v) Triton X-100 in TNE (pH 7.4), containing a standard antiprotease mix at 37°C for 1 hour.

Biotinylated proteins were captured by an overnight incubation at 4°C with streptavidin-agarose beads (Sigma-Aldrich, Germany). Supernatants, containing non-biotinylated proteins, were recovered, precipitated in 10% (w/v) TCA for 30 minutes on ice, washed twice with ice-cold acetone and resuspended in SDS-sample buffer. The pellets containing biotinylated proteins, were washed twice in TNE (pH 7.4), resuspended in sample buffer and subjected to SDS-PAGE. Proteins were then electro-transferred to nitrocellulose filters and probed with specific antibodies.

2.10 Co-immunoprecipitation

Transfected confluent cells were pulse-labeled as described above, and then cooled on ice, washed twice with ice-cold PBS and lysed on ice with RIPA buffer (150 mM NaCl, 1% (v/v) NP-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 50 mM Tris, pH 8.0), containing a standard antiprotease mix (Sigma-Aldrich, WI, USA). Cleared supernatants were recovered after centrifugation at 4°C (13,000 x g, 5 min) and incubated with different antibodies, as indicated in figure legends, at 4°C for 2 hours and then O.N. incubated with protein A-

Sepharose beads (Amersham Pharmacia, Italy). Immunoprecipitates were then collected, washed twice with ice-cold RIPA buffer (pH 8.0), resuspended with SDS-sample buffer and separated by SDS-PAGE; proteins were then electro-transferred to nitrocellulose filters and directly detected by autoradiography.

2.11 Cell treatments

2.11.1 *N*-glycosidase F treatment

Cells were seeded on 6-well plates and transfected as described above. Twenty-four hours after transfection, confluent cells were washed twice with ice-cold PBS and then incubated with 200 µl *N*-glycosidase F buffer (20 mM sodium phosphate buffer, pH 8.0, 30 mM EDTA, 0.5% (w/v) NP-40, 0.1% (v/v) SDS, 0.5% (v/v) β-mercaptoethanol) on a shaker for 20 min at 4°C. Cell lysates were scraped, collected and centrifuged at 13,000 rpm for 5 min at 4°C. After centrifugation, cleared lysates were denatured for 10 min at 90°C and then rapidly incubated O.N. at 37°C. Where indicated, samples were treated with 0.5 U *N*-glycosidase F (Sigma-Aldrich, WI, USA) during the incubation time. For SDS-PAGE analysis, 200 µl of 2X SDS-sample buffer was added. Samples were heated to 90 °C for 10 min and then loaded onto gel. Proteins were then electro-transferred to nitrocellulose filters and probed with specific antibodies.

2.11.2 Cholesterol depletion

Cells were seeded on 6-well plates and transfected as described above. Four hours after transfection, new complete medium was added in the presence or absence of 4 μM lovastatin and 0.25 mM mevalonate (Keller and Simons, 1998). After an overnight incubation at 37°C, cells treated with 10mM methyl-β-cyclodextrin for 60 min. Cells were then lysed for 20 min on ice in 0.5 ml of TNE

buffer (50 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, pH 7.5) containing 1% Triton X-100. Lysates were then collected and ultracentrifuged at 120,000 x g in TL100.1 rotor (Beckman, CA, USA) for 60 min at 4°C. Supernatants, representing the soluble material, were collected in 1.5 ml tubes and prepared for SDS-PAGE with 4X SDS-sample buffer. The pellets, representing the insoluble material, were directly resuspended in 1X SDS-sample buffer. Representative amounts of each samples were loaded on gel.

2.12 Immunofluorescence microscopy procedures

2.12.1 Materials

Alexa 488-, Alexa 546- and Alexa 633-conjugated goat anti-rabbit, antimouse, and anti-sheep antibodies, Alexa 488, Alexa 546- and Alexa 633-conjugated chicken anti-mouse and anti-rabbit antibodies were from Molecular Probes (OR, USA). Paraformaldehyde, and saponin were from Sigma-Aldrich (WI, USA). Mowiol was from Calbiochem (CA, USA).

2.12.2 List of antibodies used for morphological studies in this thesis

See Table 2.2 for the working diluitions of antibodies used in immunofluorescence experiments. In all experiments and unless indicated otherwise, MT1-MMP was immunodetected with an immuno-purified rabbit polyclonal antibody (MMR2) raised against a recombinant GST-fused polypeptide corresponding to amino acids 113-538. In all the colocalization experiments, cells were sectioned optically with z-series on a Zeiss LSM510 confocal system. Extensive overlap was observed in the perinuclear area only between MT1-MMP and furin.

Table 2.2. List of antibodies used in immunofluorescence experiments

Specificity (antibody name)	Owner (Company or Group's Head)	Animal source	Dilution
MT1-MMP (MMR2)	Our laboratory	Rabbit	1:1,000
PDI (SPA-891)	StressGen (Canada)	Mouse	1:500
Giantin	M.A. De Matteis (CMNS, Italy)	Mouse	1:1,000
TGN46	S. Ponnambalam (Leeds, UK)	Sheep	1:500
Transferrin receptor (6890)	Zymed Lab. (CA, USA)	Mouse	1:250
Mannose-6-P receptor	Affinity Bio. (CO, USA)	Mouse	1:200
VSV-G (luminal domain)	M.A. De Matteis (CMNS, Italy)	Mouse	1:1,000
FLAG (F7425)	Sigma-Aldrich (WI, USA)	Rabbit	1:200
FLAG (M1) (F3040)	Sigma-Aldrich (WI, USA)	Mouse	1:100
FLAG (M2) (F3165)	Sigma-Aldrich (WI, USA)	Mouse	1:100

2.12.3 Solutions

Paraformaldehyde: 4% (w/v) paraformaldehyde were dissolved in 65°C-heated PBS by adding a few drops of concentrated NaOH and brought to pH 7.4 with 1 M HCl. Blocking solution: 0.05 % saponin, 0.5 % BSA, 50 mM NH_4Cl in PBS. Mowiol: 20 mg of mowiol were dissolved in 80 ml of PBS, stirred O.N. and centrifuged for 30 min at 12,000 x g.

2.12.4 Procedure

Cells were fixed by adding 1 volume of 4% paraformaldehyde for 15 min at RT, and incubated in blocking solution for 60 min at RT. The cells were subsequently incubated with the specified antibodies diluted in blocking solution (see Table 2.2 for the list and dilutions of antibodies) for 2-3 hours at RT or O.N. at 4°C. After incubation with the primary antibody, cells were washed three times in PBS and incubated with a fluorescent-probe conjugated secondary antibody directed against the constant region of the primary IgG molecule for 1 hour at RT. Secondary antibodies were diluted 1:300 in blocking solution. Usually, Alexa 488- and Alexa 546- or 633- conjugated antibodies were used in double-labelling (rabbit/mouse) experiments in all possible combinations. In triple-labelling (rabbit/mouse/sheep) experiments, Alexa 488-conjugated anti-rabbit or anti-mouse antibodies raised in chicken rather than in goat were used to avoid possible crossreactions between secondary anti-sheep antibodies and secondary antibodies raised in goat. Coverslips were mounted in mowiol on microscope slides. Immunofluorescence samples were observed by a multiphoton microscope (Till photonics, Germany) or a LSM 510 confocal microscope equipped with 63X objectives (Zeiss, Germany). Optical confocal sections were taken at 1 Airy unit with resolution of 512 x 512 pixels and exported as JPEG files.

2.13 VSV-G transport assay

This assay was performed with the help of Dr. Massimiliano Baldassarre (Department of Cell Biology and Oncology, Consorzio Mario Negri Sud). A375 melanoma cells were plated on 12 mm glass coverslips, transfected with VSV-G-GFP and incubated overnight at 40°C to allow for expression while blocking exit from the endoplasmic reticulum. The following day, cells were washed 3 times with complete medium containing 20 mM HEPES and incubated at 20°C for 30 min in the same medium and chased at 32°C in the presence or absence of 0.5 %

tannic acid (Sigma-Aldrich, WI, USA). Cells were then fixed and processed for immunofluorescence as described above, with the difference that cells were not permeabilized prior to incubation with antibodies. A combination of an antibody directed against the lumenal/extracellular domain of VSV-G and a fluorophore-conjugated secondary antibody was used to detect plasma membrane staining as compared to total VSV-G measured as GFP fluorescence. For each treatment at least 20 cells were quantified. Plasma membrane staining was measured using the LSM 510 software (Zeiss, Germany) together with an electronic spreadsheet. The quantification was then normalized for cell number and expressed as a percentage of control. Experiments were repeated at least three times.

2.14 Immuno-electron microscopy procedures

These procedures were mainly performed by Dr. Galina Beznoussenko (Department of Cell Biology and Oncology – Consorzio Mario Negri Sud, Italy).

2.14.1 Ultrathin cryosectioning and immunogold labeling

Cells were fixed with 2% formaldehyde and 0.2% glutaraldehyde in 0.2 M PHEM buffer (60 mM PIPES, 25 mM HEPES, 2 mM $MgCl_2$, 10 mM EGTA, pH 7.4) washed in the same buffer and collected by centrifugation. Pellets were embedded in 10% gelatin, cooled in ice, and cut into 1 mm^3 blocks in the cold room. The blocks were infused with 2.3 M sucrose at 4°C for at least 2 hours, and frozen in liquid nitrogen. 50-60 nm-thick sections were cut at –120°C using an Ultracut R/FCS (Leica, Italy) equipped with an antistatic device and a diamond knife (Diatome, PA, USA). Ultrathin sections were picked up in a mix of 1.8 % methylcellulose and 2.3 M sucrose (1:1), used as a cryoprotectant as described (Liou et al., 1996). Cryosections were collected on formvar/carbon-coated slot copper grids and then incubated with different combinations of rabbit polyclonal antibodies followed by protein A-conjugated gold particles of different sizes (Slot

and Geuze, 1985). Double immunolabeling was performed as described before (Slot and Geuze, 1985; Webster, 1999) with optimal combinations of gold particle sizes. To reduce crossreactivity and specificity problems in double labeling experiments (when two rabbit polyclonal antibodies were used), following treatment with the first secondary antibody and protein A, sections were additionally fixed with 1% glutaraldehyde (Fluka, Sigma-Aldrich, WI, USA) for 5 min, washed with 0.12% glycine and incubated in blocking solution (0.05% saponin, 0.5% BSA, 50 mM NH_4Cl in PBS) containing 0.01% BSA-c (Aurion, The Netherlands), as previously recommended (Webster, 1999). Additionally, all sequences of primary antibody addition and combinations of protein A-conjugated gold particles of different sizes were tested. After labeling, sections were treated with 1% glutaraldehyde, counterstained with uranyl acetate, and embedded in methylcellulose/uranyl acetate as described (Slot et al., 1991). Automated data acquisition was carried out on a Tecnai 20 electron microscope at 200 kV (FEI/Philips Electron Optics, Japan) equipped with a slow-scan CCD camera.

2.14.2 Quantitative immuno-electron microscopy

To determine the distribution of MT1-MMP throughout the secretory pathway in transfected A375 cells, quantification was performed on over 30 cross-sections of cells expressing average levels of MT1-MMP. In detail, labeling density of MT1-MMP protein in different compartments was calculated as previously described (Mayhew et al., 2003), micrographs were taken at a 46,000 X magnification for each marker. Fields were taken randomly with the only criterion being a well-preserved morphology. Labeling densities in Table 4.1 were expressed as the number of gold particles per intersections of the morphometrical grid. Estimation of colocalization on EM cryosections was performed according to (Martinez-Menarguez et al., 2001).

2.15 Gelatin degradation assay

2.15.1 Preparation of fluorescent-labeled gelatin

This preparation was performed according to (Mueller and Chen, 1991), with the following modifications. Two mg/ml of gelatin were dissolved in a buffer containing 61 mM NaCl and 50mM $Na_2B_4O_7$ (pH 9.3) and then incubated at 37°C for 1 hour. After this incubation time, 1.8 mg/ml of FITC (or rhodamine) were added and mixed for 2 hours in complete darkness. This preparation was then dialysed O.N. at RT in PBS 1X in complete darkness. Dialysis was usually repeated for 2 day with 2-3 buffer changes per day. After a quick spin to remove insoluble material, small aliquots, containing 2% (w/v) sucrose, were stored in the dark at 4°C.

2.15.2 Preparation of fluorescent-labeled gelatin coated coverslips

Fluorescent gelatin coated coverslips were prepared and the assay carried out as described (Baldassarre et al., 2003; Bowden et al., 2001; Mueller and Chen, 1991). Briefly, glass coverslips were sterilized in 70% ethanol for 15 minutes at RT. Air-dried coverslips were then coated with pre-warmed fluorescent-labeled gelatin, using enough to cover the surface. Each coverslip was inverted onto a 200 ml drop of 0.5% ice-cold gluteraldehyde in PBS 1X and incubated at 4°C for 15 minutes. Each coverslip was then transferred to each well of a standard 12-well plate with coated gelatin side up, gently washed three times with PBS 1X and finally incubated with sodium borohydride (5 mg/ml) in PBS 1X for 3 minutes at RT. Washed coverslips were sterilized again in 70% ethanol for 5 minutes, dried for 20 minutes under sterile hood and then quenched in DMEM for 1 hour at 37°C. Coverslips were then ready for seeding cells.

2.15.3 Procedure

Cells were cultured on gelatin-coated coverslips for 16 hours and then fixed and processed for immunofluorescence as described above. Most experiments were analyzed with a Zeiss LSM 510 laser scanning confocal microscope (Zeiss, Germany). Immunofluorescence images were acquired at high confocality (pin hole=1 Airy unit) to achieve the thinnest possible optical slices at the substrate-cell interface. To determine the number of degrading cells for each experiment we analyzed 100 random fields (containing at least 50 transfected cells) at a 63X magnification as previously described (Baldassarre et al., 2003). In some experiments involving multi-color labeling, fluorescence was acquired by wide-field fluorescence microscopy in each channel along the *z*-axis. To remove blurring caused by the objective lens and reduce the background caused by fluorescence from out-of-focus regions, images were subjected to mathematical deconvolution with an acquired point spread function (Delta Vision, WA, USA). Images were thresholded to remove noise and enhance the contrast of structures of interest, and were superimposed and saved.

2.15.4 Quantification of gelatin degradation areas

To determine the number of degrading cells for each experiment 100 random fields (containing at least 50 transfected cells) were analyzed using a 63X objective. Values were then expressed as the percentage of transfected cells that presented at least one degradation patch (irrespective of degradation area extension) relative to the total number of cells analyzed. To determine, instead, areas of degradation, using the same criteria to select cells, the area of each degradation patch was measured using the LSM 510 software (Zeiss, Germany) together with an electronic spreadsheet. The total area for each condition was then normalized for cell number and expressed as a percentage of control. Experiments were repeated at least three times.

2.15.5 Analysis of the dynamics of gelatin degradation

This analysis was performed with the help of Dr. Massimiliano Baldassarre (Department of Cell Biology and Oncology, Consorzio Mario Negri Sud, Italy). Cells expressing MT1-MMP+GFP chimera were placed in a glass-bottom Petri dish coated with rhodamine-cojugated gelatin and containing culture medium buffered with 20 mM HEPES (pH 7.4). The Petri dish was placed on the stage of a multiphoton microscope (Till photonics, Germany) equipped with a thermoregulation device set to 37 °C. Movies were produced under a 63X oil-immersion objective, at a definition of 512x512 pixels, with the pinhole diameter set at its maximum value. The scan speed was at 1 frame/minute and, for double imaging of the same sample, the multi-track configuration was applied. Movie files were converted to TIFF format by Graphic Converter 4.0, edited by NIH Image 1.6.2, and again converted into QuickTime format.

2.16 Gelatin zymography

This method was perfomed according to (Birkedal-Hansen and Taylor, 1982), with the following modifications. Aliquots of HT1080 conditioned serum starved medium were dissolved in non-reducing SDS-sample buffer and separated by SDS-PAGE using 10% polyacrylamide gels containing 1 mg/ml of gelatin (Sigma-Aldrich, WI, USA). After electrophoresis the gels were washed twice with 50 mM Tris-HCl, pH 7.5, containing 5 mM $CaCL_2$, 1 μM $ZnCl_2$, 2.5% Triton X-100 for 15 min to remove SDS, followed by a brief rinsing in washing buffer without Triton X-100. The gels were then incubated at 37°C for 12-24 h in 50 mM Tris-HCl buffer containing 5 mM $CaCL_2$, 1 μM $ZnCl_2$, pH 7.5. The gels were then stained with coomassie brilliant blue R250 followed by destaining with 10% acetic acid, 5% methanol, as described above.

CHAPTER 3

Intracellular Trafficking and Activation of MT1-MMP

3.1 Introduction

MT1-MMP, similarly to most MMPs, is synthesized as a pro-enzyme. It is generally thought that the propeptide needs to be removed for the protein to function properly and that this occurs through cleavage at a short amino acid stretch at the C-terminus of the propeptide by the action of a prohormone convertase (furin or similar, Sato et al., 1996; Yana and Weiss, 2000). It has also been proposed, however, that MT1-MMP does not require the cleavage of its propeptide to enable it to activate MMP-2 in certain cell types (Cao et al., 1996). In addition, activation of MT1-MMP might also occur by plasmin at the cell surface (Okumura et al., 1997). A further layer of complexity derives from the fact that mechanisms as diverse as autocatalytic processing, ectodomain shedding, homodimerization and internalization might all contribute to the modulation of MT1-MMP activity on the cell surface (for a review, Osenkowski et al., 2004). Indeed, MT1-MMP has also been found to be partially located in detergent-resistant membrane (DRM) fractions (Annabi et al., 2001), and recent results have addressed the role of DRM in MT1-MMP/CD44/caveolin interaction and hyaluronan cell surface binding (Annabi et al., 2004). Hence, MT1-MMP membrane partitioning could represent a mechanism for the regulation of its activities. At variance with these findings, it has been recently proposed that only a deletion mutant of MT1-MMP lacking the cytosolic C-terminal tail is partitioned into DRM (Rozanov et al., 2004a).

The intracellular processing and activation of MT1-MMP, and their

dependence on partitioning within different membrane subdomains are the subject of this results chapter.

3.2 Results

3.2.1 MT1-MMP processing and membrane partitioning

To analyze MT1-MMP, I produced two new rabbit polyclonal antisera (MMR1, MMR2) raised against the same GST-fused polypeptide corresponding to amino acids 113-538 of MT1-MMP (see section 2.6 in Chapter 2). These were tested and compared to the commercially available affinity-purified polyclonal antibody M3927 by immunoprecipitation from untransfected and MT1-MMP-transfected A375 melanoma cells (Fig. 3.1 A). The three different antibodies displayed uneven efficiency in the detection of a lower molecular mass (60 kDa) MT1-MMP band, presumably the fully processed mature form. To better characterize the 60 kDa MT1-MMP band, I produced another rabbit polyclonal antiserum (MMR3), raised against a GST-fused polypeptide corresponding to the propeptide domain of MT1-MMP (amino acids 35-112), as a tool for the specific detection of unprocessed immature MT1-MMP forms. MMR2 and MMR3 antibodies were tested by immunoprecipitation from MT1-MMP-transfected A375 melanoma cells (Fig. 3.1 B). By comparing the molecular patterns of MT1-MMP detected with these two different antibodies, I could definitively concluded that the 60 kDa band corresponds to the propeptide-deleted MT1-MMP form.

This set of data is of great practical importance because: 1) it suggests that detection of mature MT1-MMP is generally quite problematic as exemplified comparing the antisera generated in different animals but raised against the same polypeptide (MMR1 and MMR2); 2) the interpretation of experimental data on the maturation of MT1-MMP obtained with different antibodies (MMR2 and MMR3) becomes a critical issue for the interpretation of these results.

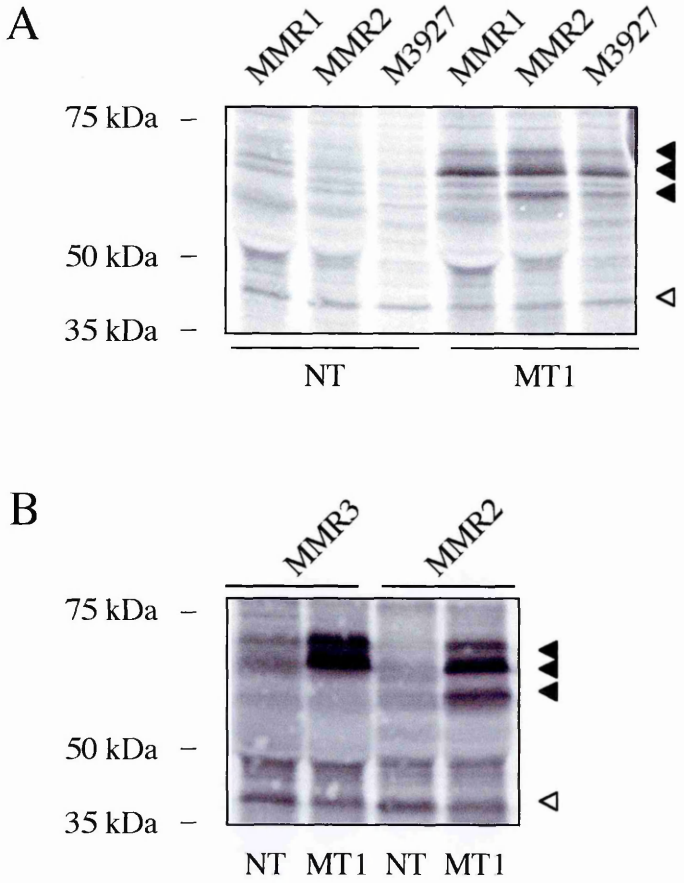


Fig. 3.1. Molecular characterization of MT1-MMP processing in A375 melanoma cells using different anti-MT1-MMP antibodies. Untransfected (NT) and MT1-MMP-transfected (MT1-MMP) A375 cells were pulse-labeled with [^{35}S]methionine/cysteine for 2 hours and their cleared lysates were immunoprecipitated with different antibodies: (A) MMR1 and MMR2, rabbit polyclonal antisera raised against the same GST-fused polypeptide corresponding to amino acids 113-538 of MT1-MMP; M3927, affinity-purified polyclonal antibody. (B) MMR3, rabbit polyclonal antiserum raised against a GST-fused polypeptide corresponding to amino acids 34-112 of MT1-MMP. MT1-MMP immunoprecipitates in A and B were subjected to SDS-PAGE, transferred to nitrocellulose membrane and visualized by autoradiography. The black arrowheads mark the positions of the MT1-MMP forms detected: the calculated molecular weights are, respectively, 65, 63 and 60 kDa. The positions of molecular weight standards are indicated on the left. The white arrowhead marks an unspecific band visible in all lanes which should not be confused with the 43 kDa form shown to be a MT1-MMP degradation product in some reports (see Introduction).

Since MMR2 appeared to be by far the most sensitive antibody, it was used in all subsequent immunoprecipitation experiments. To study MT1-MMP processing, MT1-MMP-transfected A375 cells were pulse-labeled with [^{35}S]methionine/cysteine for 5 minutes and, after different chase times, processed for immunoprecipitation with MMR2. Three molecular forms of MT1-MMP could be detected of 65, 63 and 60 kDa (Fig. 3.2).

The 63 kDa form (also called proMT1-MMP) had a slow turnover rate over a time frame of 2 hours; only a minor fraction was converted to the mature lower molecular mass 60 kDa form, which instead appeared to turn over much more rapidly (Fig. 3.2). When BB94, a broad-range metalloprotease inhibitor (Davies et al., 1993), was included during preincubation and chase, MT1-MMP maturation occurred normally (see last lane in Fig. 3.2) excluding that, at least in my experimental conditions, MT1-MMP was functioning as a self-convertase, as proposed by one group (Rozanov and Strongin, 2003).

When treatments affecting transport or processing are applied such as the fungal toxin Brefeldin A (BFA), the 65 kDa form becomes dominant (Fig. 3.3). BFA treatment leads to intermixing between the endoplasmic reticulum and the Golgi apparatus and blocks exit from the Golgi apparatus itself (Fujiwara et al., 1988; Lippincott-Schwartz et al., 1989). Proteins normally in transit through the endoplasmic reticulum and the Golgi apparatus would thus be over modified by different enzymes enriched in those compartments (e.g. glycosyltransferases), due to an increased exposure. For this reason, the 65 kDa form, previously observed (Maquoi et al., 1998; Yana and Weiss, 2000), has been proposed to be a post-translationally highly modified unprocessed immature form (Maquoi et al., 1998). To test this directly, I performed a deglycosylation assay using *N*-glycosidase F, which removes glycosylations aspecifically. After a 20 hours treatment of the cell lysates at 37°C in a sodium phosphate buffer, I verified no changes in the steady-state biochemical pattern of MT1-MMP if compared to the hyper-glycosylated human α1-antitrypsin mutant, α1-PDX, used here only as

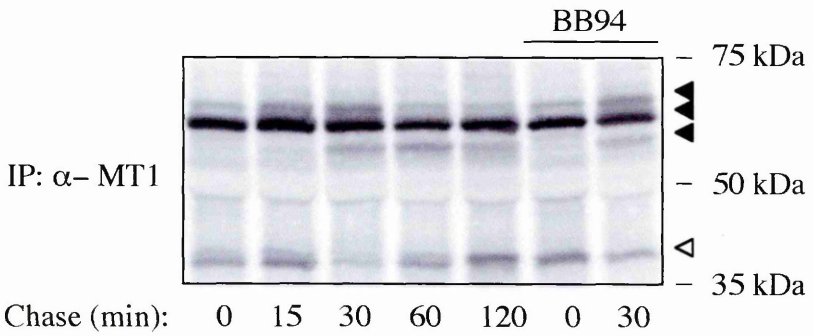


Fig. 3.2. Time-course analysis of MT1-MMP processing. MT1-MMP-transfected A375 cells were pulse-labeled with [^{35}S]methionine/cysteine for 5 minutes and chased for the indicated times. Where indicated, transfected cells were preincubated with 5 μM BB94 overnight and throughout the chase and immunoprecipitated with anti-MT1-MMP antiserum MMR2. Immunoprecipitates in A and B were subjected to SDS-PAGE, transferred to nitrocellulose membrane and visualized by autoradiography. The black arrowheads mark the positions of the MT1-MMP forms detected: calculated molecular weights are, respectively, 65, 63 and 60 kDa. The white arrowhead marks an unspecific band visible in all lanes. The positions of molecular weight standards are indicated on the right.

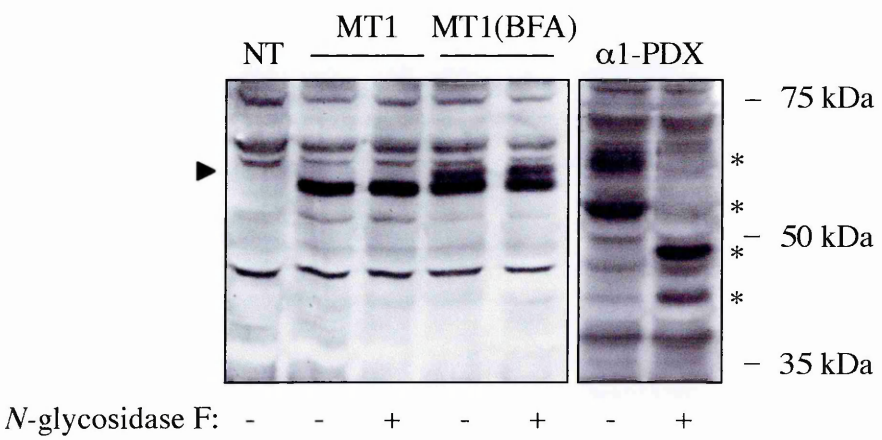


Fig. 3.3. Biochemical characterization of the 65 kDa MT1-MMP form. Untransfected (NT), MT1-MMP-transfected (MT1) and α1-PDX-transfected (α1-PDX) A375 cells were lysed in a sodium phosphate buffer and processed, where indicated, with *N*-glycosidase F at 37°C for an overnight incubation, as described in Materials and Methods. Cell lysates were then subjected to SDS-PAGE, transferred to nitrocellulose membrane and visualized by Western blotting with an immuno-purified rabbit polyclonal anti-MT1-MMP antibody (M3927) or with a rabbit polyclonal antiserum raised against human a1-antitrypsin (Dako, Denmark). Where indicated, MT1-MMP-transfected cells were treated with 2.5 μg/ml BFA for 2 hours before lysis. The black arrowhead marks the positions of the 65 kDa MT1-MMP form detected upon BFA treatment. The asterisks mark differently glycosilated specific bands detected for α1-PDX. The positions of molecular weight standards are indicated on the right.

a positive control. This is taken to indicate that the BFA-sensitive upper band is not hyper-glycosylated (Fig. 3.3). I have not further characterized this protein band as it is beyond the scope of my thesis.

Both endogenous and overexpressed MT1-MMP has been recently described to be partially located in detergent-resistant membranes (Annabi et al., 2001). Insolubility in detergents is typical of glycolipid-rich domains, also referred to as "lipid rafts" (Simons and Ehehalt, 2002). It was thus possible that two populations of MT1-MMP, one associated with insoluble and the other with soluble membrane fractions, could coexist in transfected A375 cells and explain its peculiar processing pattern. I tested the Triton X-100 solubility of MT1-MMP in total membrane preparations from MT1-MMP-transfected A375 melanoma cells and found that the majority of MT1-MMP (at least 70-80%) was Triton X-100-insoluble (P) and unprocessed (see 63 kDa band; Fig. 3.4). To exclude the possibility that insolubility was due to association with cytoskeletal elements or to the formation of non-specific protein aggregates, I further verified the distribution of MT1-MMP on an Optiprep-density gradient as described in Materials and Methods. I found that the majority of the 63 kDa proMT1-MMP floated towards the top of the gradient (Fig. 3.5 A), confirming that this population of proMT1-MMP was associated with authentic detergent-insoluble membranes. The assay was validated by testing the distribution of caveolin-1, a bona-fide marker for raft-type membrane domains, and which co-fractionated with proMT1-MMP (Fig. 3.5 B). Again, only the unprocessed 63 kDa proMT1-MMP was detected in the DRM-enriched fraction (lane 5 in Fig. 3.5 A).

3.2.2 Divergent processing of MT1-MMP depending on its partitioning in different membrane subdomains

The above data suggested the possibility that differential MT1-MMP processing was connected to its partitioning in different membrane subdomains.

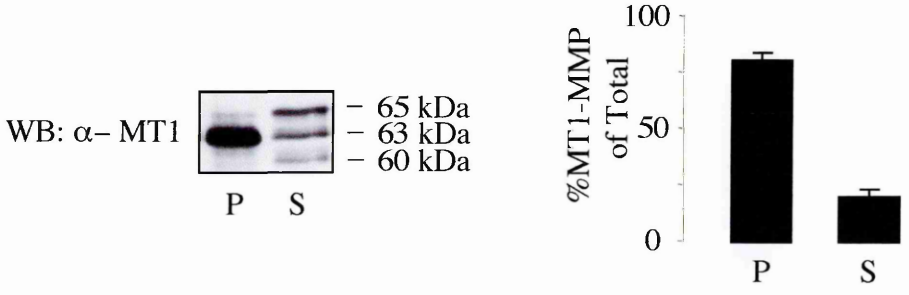


Fig. 3.4. MT1-MMP localization in detergent-resistant membranes (DRM). MT1-MMP transfected A375 cells were lysed with 1% Triton X-100-containing TNE buffer, as described in Materials and Methods. Insoluble (P) and soluble (S) fractions were separated by ultracentrifugation at 4°C (120,000 x g, 1 hour), resuspended in sample buffer and subjected to SDS-PAGE, transferred to nitrocellulose membrane and visualized by Western blotting with the MMR2 antibody. The amount of 63 kDa form in each fraction was determined with Fuji BAS software and plotted in the bar chart.

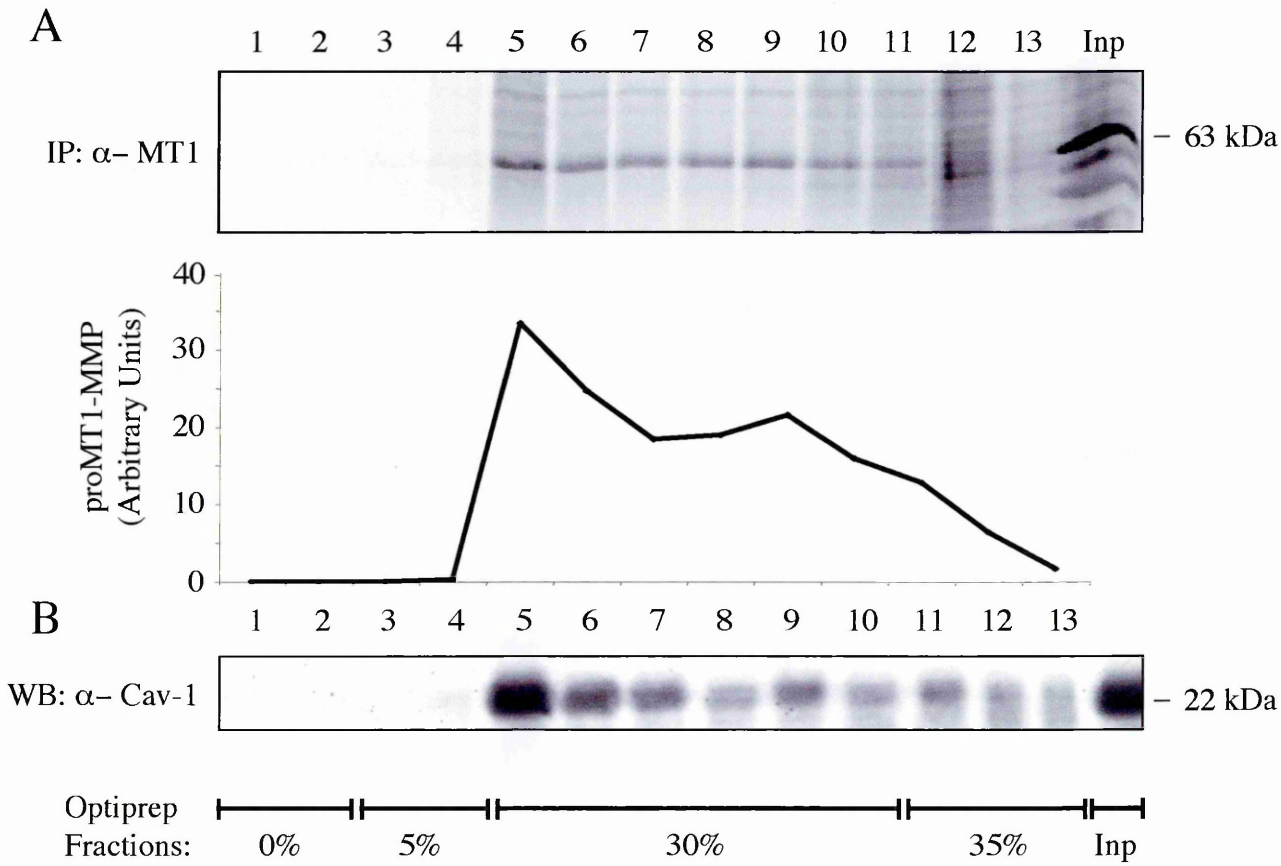


Fig. 3.5. MT1-MMP localization in low-density Optiprep-gradient fractions from A375 melanoma cells. (A) MT1-MMP-transfected A375 cells were labeled with [^{35}S]methionine/cysteine for 2 hours and lysed with a 1% Triton X-100-containing buffer, as described in Materials and Methods. Lysates were fractionated on a discontinuous Optiprep-density gradient, as reported in the scheme on the bottom. MT1-MMP immunoprecipitates from each fraction and a sample of the initial preparation (Inp) were subjected to SDS-PAGE, transferred to nitrocellulose membrane and visualized by autoradiography. The amount of 63 kDa form in each fraction was determined with Fuji BAS software and plotted graphically. (B) A small sample of each fraction was tested by Western blotting with a polyclonal anti-caveolin-1 antibody as a bona-fide marker for the DRM preparation.

To test this hypothesis, MT1-MMP-transfected cells were pulse-labeled with [^{35}S]methionine/cysteine for 5 minutes and, after different chase times, lysed with 1% Triton X-100. Soluble (S) and insoluble (P) fractions were separated by ultracentrifugation and immunoprecipitated with polyclonal antibody MMR2 (Fig. 3.6). The detergent-soluble 63 kDa proMT1-MMP fraction is rapidly processed to the mature form within 20 minutes and, after 60 minutes, is greatly reduced (see lanes S in Fig. 3.6). As a whole, the soluble MT1-MMP pool undergoes a rapid turnover (Fig. 3.6) and the mature MT1-MMP pool is degraded starting from 2 hours (Fig. 3.2); DRM-associated proMT1-MMP, instead, is stable and remains unprocessed even after a 2 hour-long chase (Fig. 3.2). This indicates that only the detergent-soluble pool of MT1-MMP undergoes maturation and rapid degradation; DRM-associated proMT1-MMP is, on the other hand, completely excluded from processing and is quite stable over time.

3.2.3 Characterization of MT1-MMP association with detergent-resistant membranes

Cholesterol is a crucial structural component of lipid rafts and is enriched in DRM (Brown, 1993; Simons and Ikonen, 1997). To directly demonstrate the involvement of cholesterol, I decided to analyse Triton X-100 insolubility of MT1-MMP in cholesterol-depleted membranes by using methyl-β-cyclodextrin (mCD), previously described as a powerful tool for extracting cholesterol from biological membranes (Klein et al., 1995). At least in my experimental conditions, however, mCD does not seem to affect MT1-MMP partitioning in different membrane fractions extracted from MT1-MMP-transfected A375 cells (Fig. 3.7). It must be noted that mCD treatment was performed acutely on MT1-MMP-transfected A375 cells and then MT1-MMP was analysed by performing immunoblotting analysis at steady-state. This is neither the most sensitive nor the sole approach to detect possible changes in the partitioning of a protein in

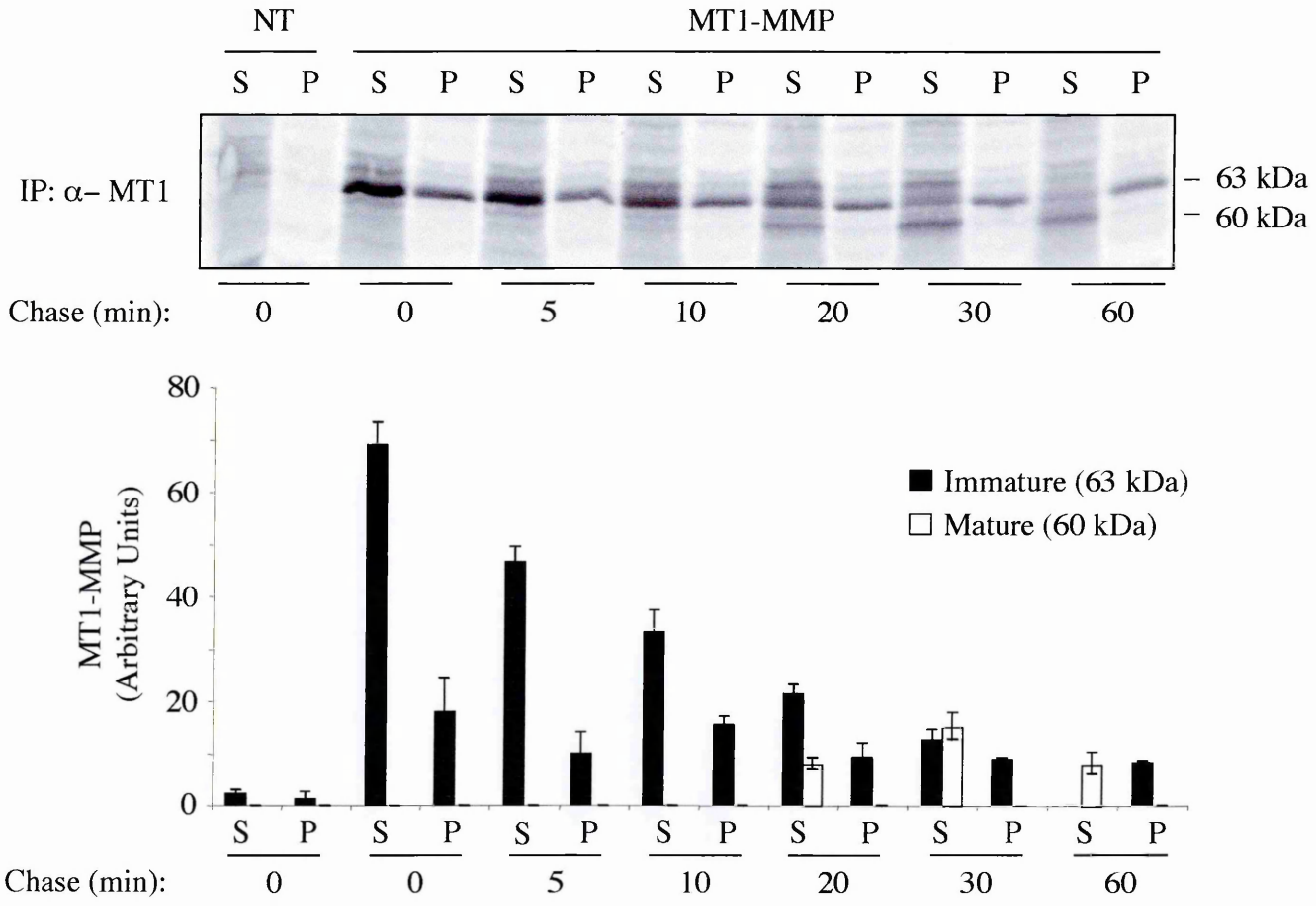


Fig. 3.6. Time-course analysis of MT1-MMP processing in A375 melanoma cells and association with different membrane fractions. Untransfected (NT) and MT1-MMP-transfected A375 cells were pulse-labeled with [^{35}S]methionine/cysteine for 5 minutes and, after the indicated chase times, incubated with TNE containing 1% Triton X-100 on ice. Soluble (S) and insoluble (P) fractions were separated by ultracentrifugation, immunoprecipitated with polyclonal anti-MT1-MMP antiserum MMR2, subjected to SDS-PAGE, transferred to nitrocellulose membrane and visualized by autoradiography. The bar chart shows the distribution in time of immature and mature MT1-MMP and association with different membrane fractions as determined with the Fuji BAS software.

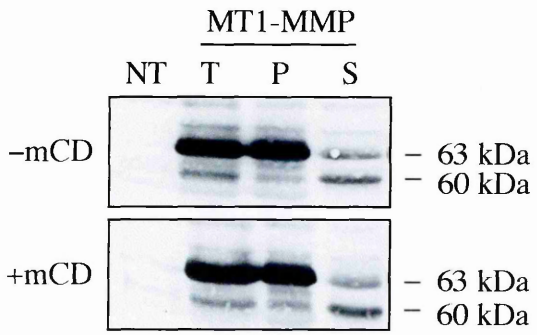


Fig. 3.7. Cyclo-β-methyldextrin does not affect MT1-MMP partitioning within different membrane fractions. Untransfected (NT) and MT1-MMP-transfected A375 cells were treated, where indicated, with 10 mM cyclo-β-methyldextrin (mCD) for 60 min at 37°C, as described in Materials and Methods. Cells were then lysed with 1% Triton X-100-containing TNE buffer, as described in Materials and Methods. Insoluble (P) and soluble (S) fractions were separated by ultracentrifugation at 4°C (120,000 x g, 1 hour), resuspended in sample buffer and subjected to SDS-PAGE, transferred to nitrocellulose membrane and visualized by Western blotting with the MMR2 antibody. A small amount of total lysates (T) were also loaded. The effect of cyclo-β-methyldextrin on A375 cells was monitored by phase-contrast microscopy.

cholesterol-rich membranes; other biophysical conditions might confer detergent insolubility to MT1-MMP; for instance, oligomerization of MT1-MMP itself (Lehti et al., 2002) or MT1-MMP recruitment by other proteins, such as caveolin-1, that can bind tightly to cholesterol (Murata et al., 1995). In this context, the question of whether or not the MT1-MMP pool associated with detergent-resistant membranes is indeed located within "canonical" lipid rafts might have important implications with regard to the mechanisms involved in the regulation of its activity. For this reason, I pulsed MT1-MMP-transfected A375 cells with [^{35}S]methionine/cysteine for 5 minutes (instead of 2 hours as in Fig. 3.5), and I placed the membrane extracts at the bottom of a discontinuous Optiprep gradient to verify whether this newly synthesized MT1-MMP floats to the top of the gradient. Figure 3.8 shows that, in this case, the snap-pulsed proMT1-MMP pool is mainly associated with the high-density fractions, although a small proportion is already associated with detergent-insoluble membranes (see Fig. 3.6).

Insolubility in Triton X-100 is a characteristic of "detergent-resistant membranes" (DRM), of which "lipid rafts", defined as low-density structures enriched in cholesterol and sphingolipids, can be considered only a subclass (Helms and Zurzolo, 2004). In the specific case of MT1-MMP, DRM association seems to occur early and soon after protein synthesis within the endoplasmic reticulum and prior to the enrichment in esterified lipids within the Golgi stacks, a general indicator for lipid raft maturation (Khelef et al., 2000). Subsequent lipid enrichment would be correlated with MT1-MMP flotation within low-density Optiprep fractions. These data would be consistent with recent evidence that association of glycosylphosphatidylinositol (GPI)-anchored proteins with "lipid-enriched" DRM occurs concomitantly with their oligomerization within the trans-Golgi network (Paladino et al., 2004). This speculation is discussed with details later (see section 3.3.2).

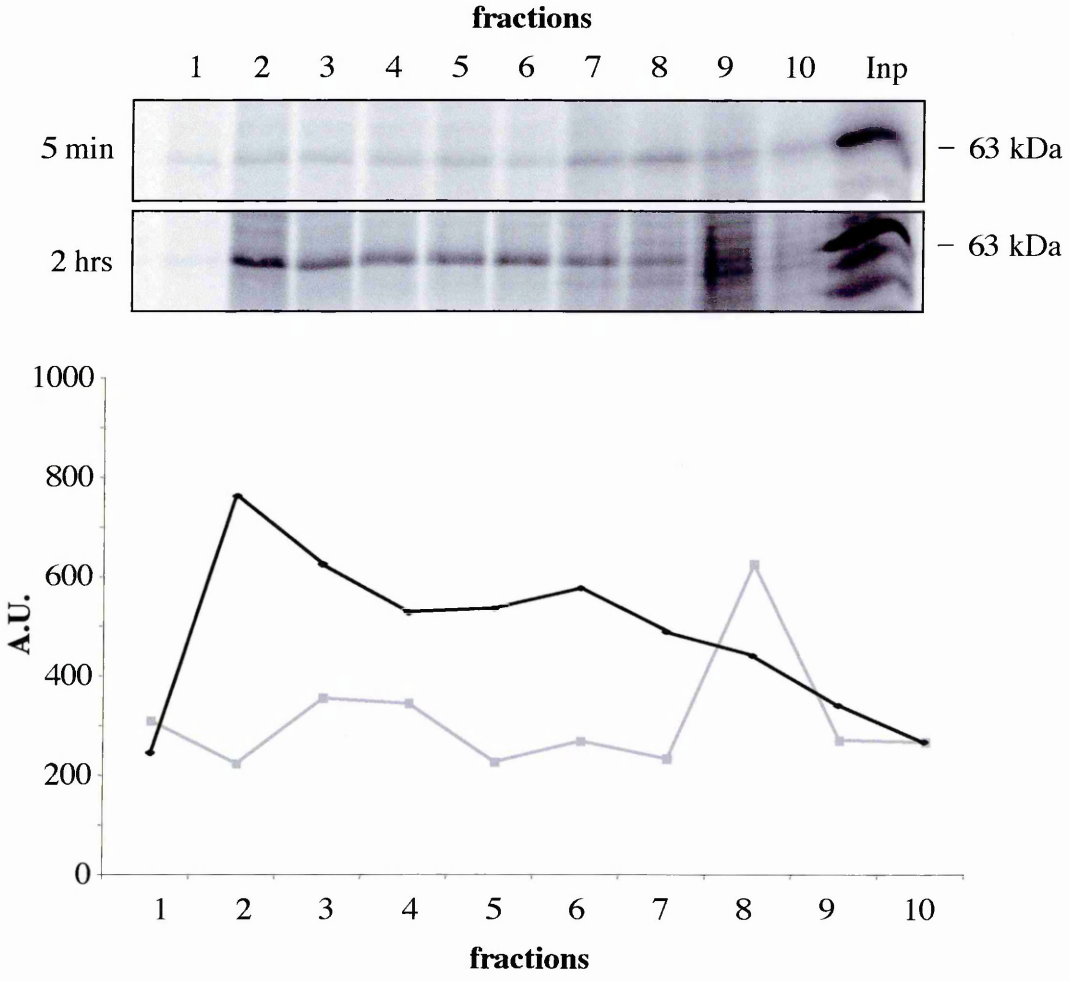


Fig. 3.8. Biochemical characterization of proMT1-MMP association with DRM. MT1-MMP-transfected A375 cells were labeled with [^{35}S]methionine/cysteine for 5 min and 2 hrs, as indicated, and lysed with a 1% Triton X-100-containing buffer, as described in Materials and Methods. Lysates were fractionated on a discontinuous Optiprep-density gradient, as in Fig. 3.5. MT1-MMP described immunoprecipitates from each fraction and a sample of the initial preparation (Inp) were subjected to SDS-PAGE, transferred to nitrocellulose membrane and visualized by autoradiography. The normalized amount of 63 kDa form in each fraction was determined with Fuji BAS software, expressed as arbitary units (A.U.) and plotted graphically in grey (5 min) and black (2 hrs).

3.2.4 Functional role of different MT1-MMP forms in ECM degradation

Overexpression of MT1-MMP in A375 cells led to, as expected, a massive degradation of cross-linked fluorescent gelatin. When monitored *in vivo* through the use of a GFP-tagged MT1-MMP chimera (which produces the same phenotype as wild-type MT1-MMP), this appeared to be an extremely rapid process (see time-lapse series in Fig. 3.9). The question thus arises as to which molecular forms and populations of MT1-MMP are responsible for this biological activity. A number of experiments were thus performed to better understand the processing of MT1-MMP and define the molecular form(s) responsible for the biological activity, a controversial issue in the field.

To interfere with proMT1-MMP processing, I decided to use the serpin-like protein α1-PDX, a bioengineered α1-antitrypsin characterized as an extremely specific endocellular inhibitor of the pro-protein convertases furin and PC6 (Jean et al., 1998): expression of α1-PDX completely abated MT1-MMP-induced gelatin degradation by A375 cells (Fig. 3.10). Gelatin degradation relies on cell surface presentation of enzymatically active MT1-MMP; I thus verified the presence and membrane partitioning of MT1-MMP at the plasma membrane whether or not proMT1-MMP processing is inhibited by α1-PDX. To this end, I performed biotinylation of surface proteins, as described in the material and methods section. Surface proteins were biotinylated on ice (to block all membrane trafficking events) and then probed by Western blotting analysis of the different membrane fractions (detergent-resistant vs. detergent-soluble) and cellular locations (intracellular vs. surface). I observed that, at steady state, the vast majority of MT1-MMP was intracellular, immature and associated with DRM (Fig. 3.11). Although total plasma membrane MT1-MMP levels were relatively low, probably because surface biotinylation is not very efficient, DRM-associated proMT1-MMP remained the dominant fraction over non-DRM proMT1-MMP.

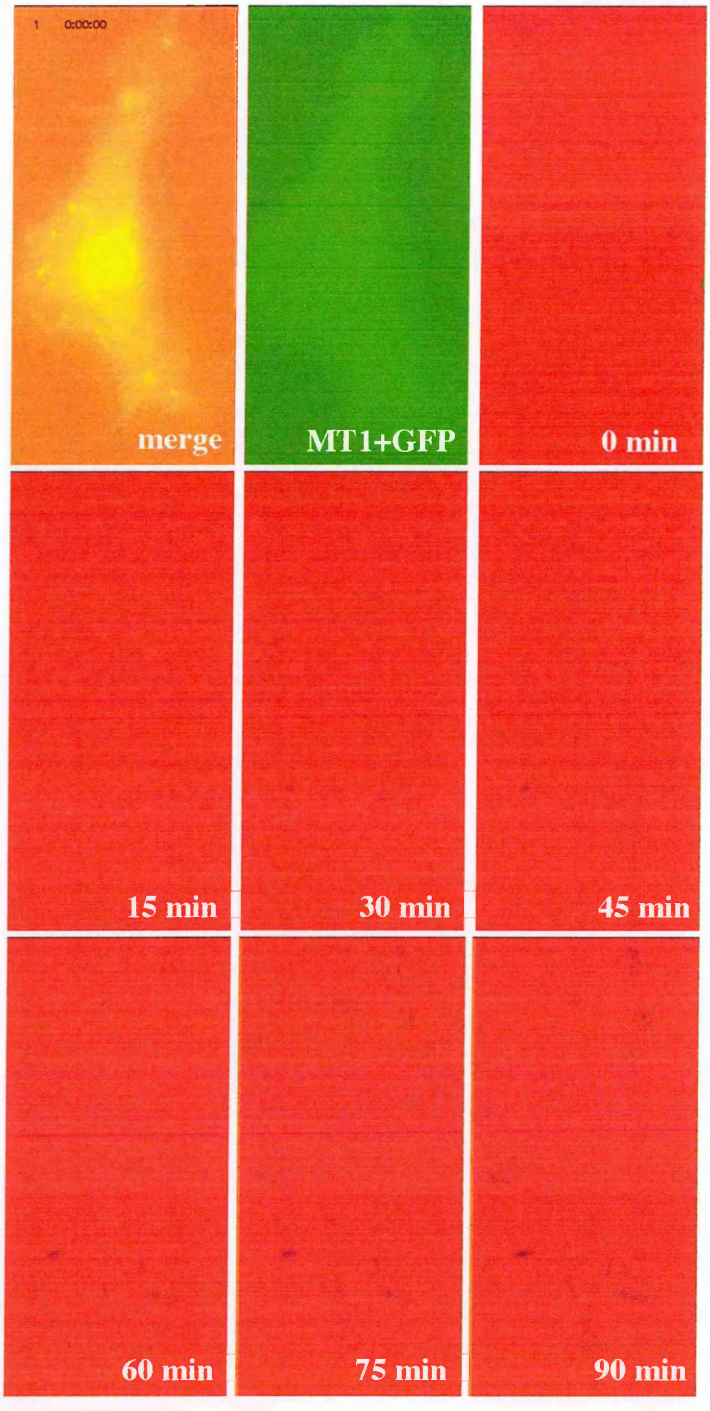


Fig. 3.9. Time-lapse series of gelatin degradation by A375 melanoma cells transfected with GFP-tagged MT1-MMP. Cells were cultured in glass bottom microwell dishes (Mattek, Ashland, MA, USA) coated with rhodamine-conjugated gelatin in the presence of 5 μM BB94 to block MMP activity. After 16 hours, cells were washed 3 times to remove BB94 and incubated in DMEM/F12 containing 20 mM HEPES pH 7.4 on a multiphoton microscope (Till photonics, Germany) fitted with a CCD camera. Images were acquired at 1 frame/minute for 1.5 hours. Representative images are shown here with indicated times. This experiment was performed in collaboration with Massimiliano Baldassarre (Consorzio Mario Negri Sud).

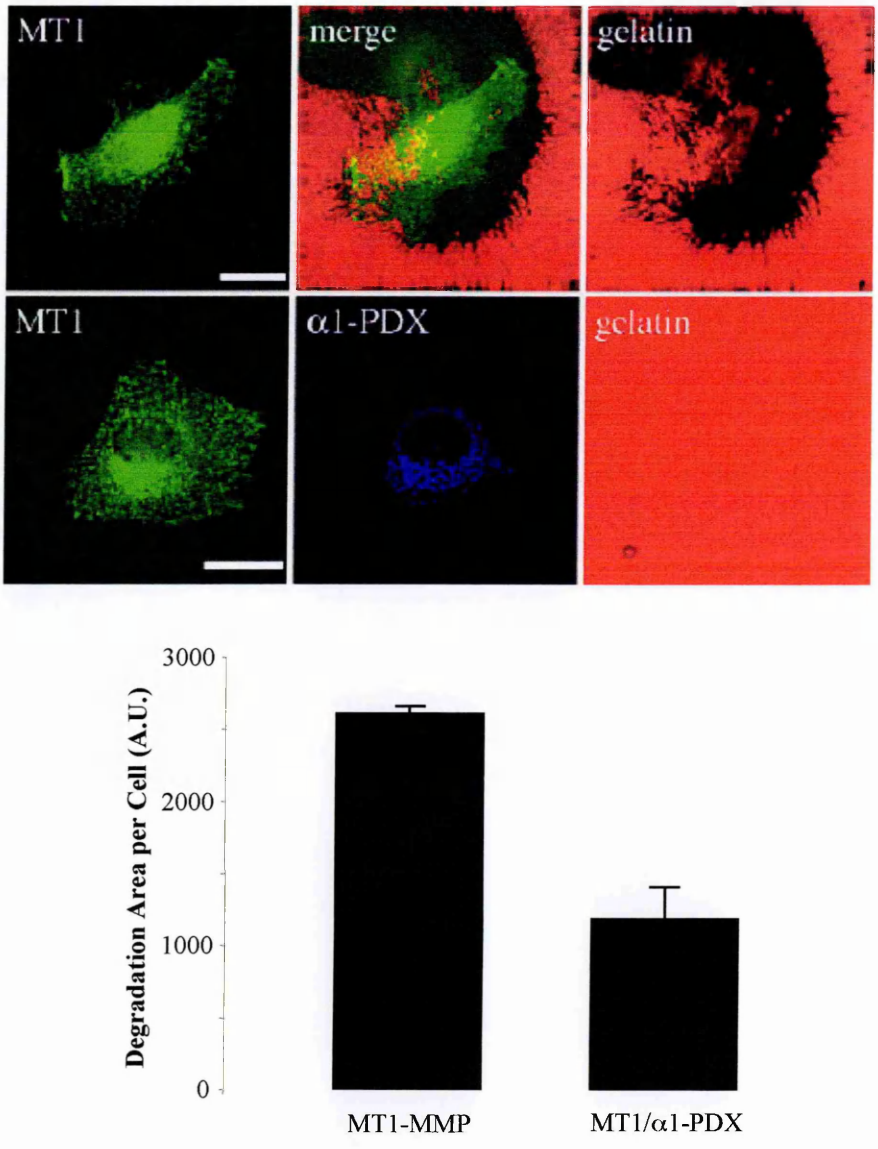


Fig. 3.10. α1-PDX blocks gelatin degradation in MT1-MMP-transfected A375 melanoma cells. A375 cells transfected with MT1-MMP and pcDNA 3.0 (MT1) or MT1-MMP and FLAG-tagged α1-PDX (MT1/α1-PDX) were cultured on surfaces coated with rhodamine-conjugated gelatin. After 16 hours of incubation, cells were fixed and labeled with specific primary antibodies: polyclonal immunopurified anti-MT1-MMP antibody MMR2 (MT1) and monoclonal anti-FLAG antibody M2 (α1-PDX). Degradation of the underlying fluorescent gelatin by the MT1-MMP/pcDNA3.0 and MT1-MMP/α1-PDX cotransfectants is shown (Gelatin). Merged staining (MT1-MMP/Gelatin) for the MT1-MMP/pcDNA3.0 cotransfectants is also shown. Transfected cells were visualized by wide-field fluorescence microscopy and acquired images were deconvoluted as described in Materials and Methods. The graph is a quantification of the average degradation area per single cell as determined using with NIH ImageJ 1.32 software and expressed in arbitrary units. Scale bars: 20 μm.

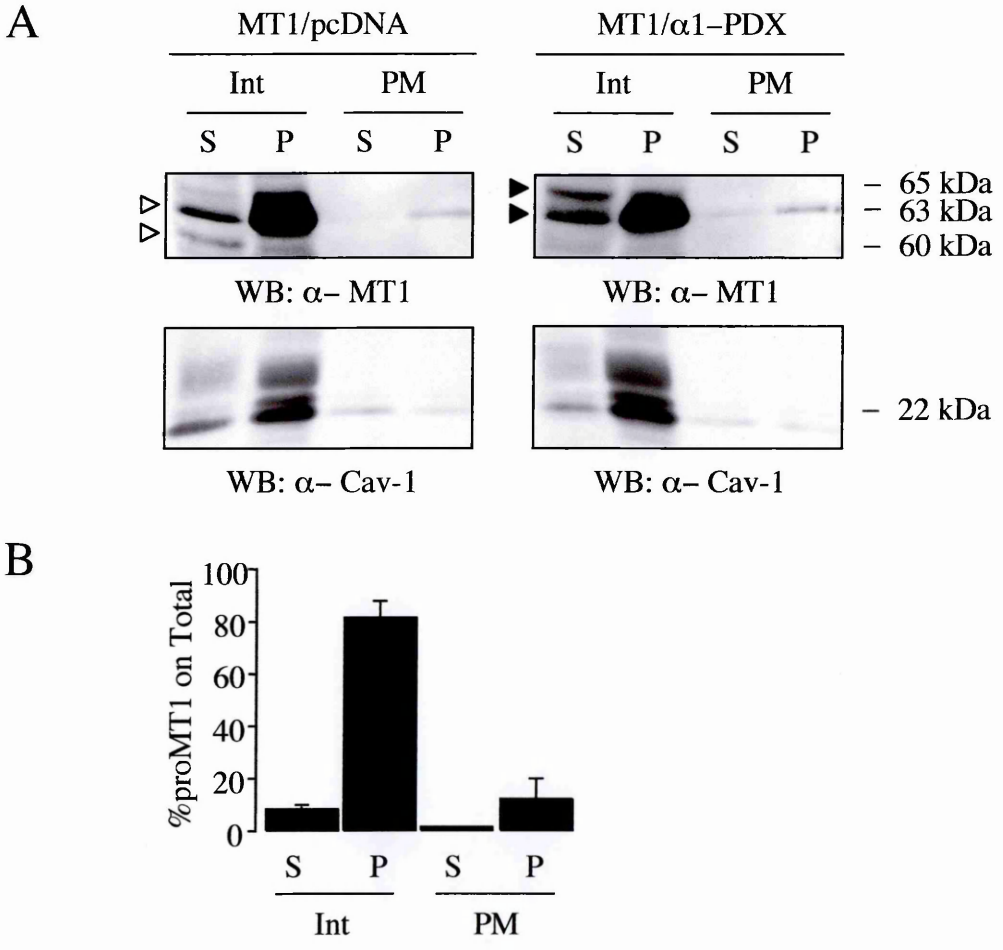


Fig. 3.11. α1-PDX blocks proMT1-MMP processing but does not affect cell surface presentation of DRM-associated proMT1-MMP. (A) Steady-state distribution profile of MT1-MMP in A375 melanoma cells: A375 cells transfected with MT1-MMP and pcDNA3.0 or MT1-MMP and α1-PDX were biotinylated and incubated with TNE containing 1% Triton X-100 on ice. Soluble (S) and insoluble (P) fractions were separated by ultracentrifugation. Intracellular fractions (Int) were separated from plasma membrane fractions (PM) by capturing the biotinylated proteins with streptavidin-conjugated agarose beads. Each fraction was subjected to SDS-PAGE and transferred on nitrocellulose membrane. The MT1-MMP distribution profile was analyzed with immunopurified polyclonal anti-MT1-MMP antibody MMR2. The empty arrowheads mark the positions of the pro- and mature (63 and 60 kDa) forms in the MT1-MMP/pcDNA cotransfectants, the black arrowheads indicate the 65 and 63 kDa immature forms in the MT1-MMP/α1-PDX cotransfectants. Staining for caveolin-1 in the same experiment is reported as a bona-fide marker for the DRM preparation. (B) The graph shows the steady-state distribution profile of proMT1-MMP within different membrane fractions: soluble (S), insoluble (P), intracellular (Int) and plasma membrane fractions (PM). The values were determined with the NIH image software.

This evidence also implies that mature 60 kDa MT1-MMP, shown here to be associated with the detergent-soluble membranes, is rapidly removed from the cell surface after presentation. This is in good agreement with the rapid turnover of MT1-MMP as verified in our pulse-chase analysis (see Fig. 3.6).

I then proceeded to analyze the effect of furin inhibition on MT1-MMP partitioning at the plasma membrane: although co-expression with α1-PDX totally inhibited the proteolytic processing of detergent-soluble MT1-MMP (Fig. 3.10), proMT1-MMP was still present at the plasma membrane (Fig. 3.11). Interestingly, in these conditions an increase of the 65 kDa unprocessed form of MT1-MMP was observed. As noted above, this has been previously reported (Maquoi et al., 1998; Yana and Weiss, 2000) and also occurs when treatments affecting transport or processing are applied such as Brefeldin A (see Fig. 3.3).

In conclusion, these results clearly indicate that: 1) only the detergent soluble proMT1-MMP fraction is processed to the mature 60 kDa form; 2) this fully processed MT1-MMP alone is responsible for gelatinolytic activity; 3) the processing enzyme is most likely furin or a furin-like convertase as previously suggested and 4) removal of the propeptide domain is not a prerequisite for arrival of MT1-MMP at the plasma membrane.

3.2.5 Furin is excluded from DRM

My data indicates that furin, the MT1-MMP activating enzyme, is unable to process DRM-associated MT1-MMP which, as a result, reaches the plasma membrane in its 63 kDa immature form. A possible explanation for the inability of furin to activate the large fraction of DRM-associated MT1-MMP could be that they are physically segregated during transport and thus unable to interact. To test this, I examined the membrane partitioning of a GFP-tagged furin chimera by transient co-transfection with MT1-MMP. As shown in Fig. 3.12 A, despite the increased cellular burden due to overexpression, furinGFP, in contrast to MT1-MMP, was exclusively associated with the detergent-soluble membrane fraction.

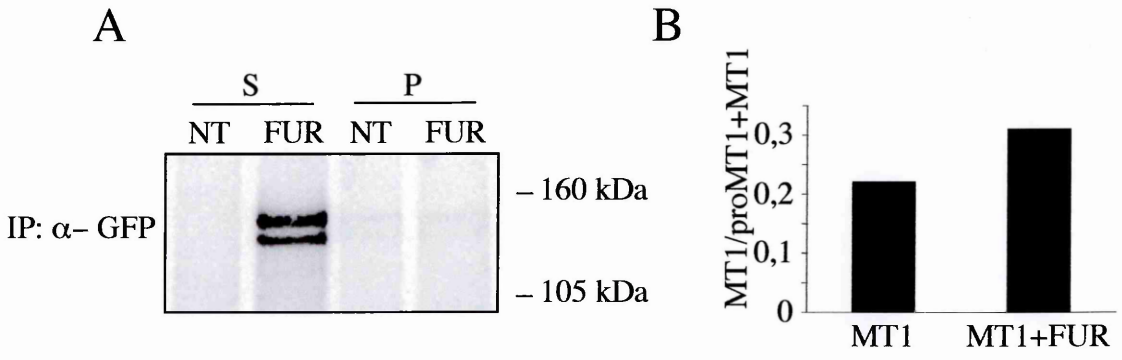


Fig. 3.12. Furin is excluded from detergent-resistant membranes. (A) Untransfected (NT) and Furin-GFP-transfected (FUR) A375 cells were labeled with [^{35}S]methionine/cysteine for 2 hours and lysed with TNE buffer containing 1% Triton X-100 on ice. Soluble (S) and insoluble (P) fractions were separated by ultracentrifugation, immunoprecipitated with a polyclonal anti-GFP antibody and analyzed by SDS-PAGE/autoradiography. (B) A375 cells transfected with MT1-MMP (MT1-MMP) or with MT1-MMP and Furin-GFP (MT1+FUR) were labeled with [^{35}S]methionine/cysteine for 2 hours and lysed with TNE containing 1% Triton X-100 on ice. Soluble fractions were collected after ultracentrifugation, immunoprecipitated with polyclonal anti-MT1-MMP antiserum MMR2 and analyzed by SDS-PAGE/autoradiography. The processing ratio of MT1-MMP (MT1-MMP/MT1-MMP+proMT1-MMP) was determined with the Fuji BAS software and plotted graphically.

GFP chimeras have been extensively used to study membrane partitioning of diverse proteins with no reported alterations in normal profiles (Rodgers, 2002). Furthermore, in my experimental system, the furin-GFP chimera was enzymatically active as its expression clearly increased the extent of MT1-MMP maturation (Fig. 3.12 B).

Taken together, these results further confirm that partitioning of MT1-MMP into different membrane sub-domains regulates its processing and biological activities. This also supports the notion that differential partitioning into DRM reflects an intrinsic property of MT1-MMP similarly to the Alzheimer precursor protein (Ehehalt et al., 2003).

3.2.6 Functional role of different MT1-MMP forms in MMP-2 activation

3.2.6.1 Background

I have thus far described experiments in A375 melanoma cells revealing that MT1-MMP undergoes biochemical processing only if associated with non-detergent-resistant membrane fractions. I was also able to confer a functional meaning to this soluble processed MT1-MMP form: it is catalytically active and responsible for gelatin degradation.

A375 melanoma cells, selected as a model cell system, normally present a basal MMP-dependent degradation only in complete medium; this is probably dependent on the basal activation of soluble MMPs (e.g. MMP-2) present in the serum. Overexpression of MT1-MMP directly confers to these cells an extremely aggressive gelatin degradation ability with or without serum, as verified by *in vitro* gelatin degradation assays (see introduction). This activity can be totally inhibited upon overexpression of α1-PDX, a selective furin inhibitor, as a consequence of the total inhibition of proMT1-MMP processing (see Fig. 3.10).

Therefore, my data bring further evidence that furin is a key factor in the maturation of MMPs associated with the invasive and metastatic potential of tumor cells, as reported in literature (Maquoi et al., 1998; Yana and Weiss, 2000). This scenario, however, could be more complicated depending on both the specific protein expression profiles of the tumor cells used and the extracellular environment tested.

MT1-MMP was initially reported to be expressed in carcinoma cells in addition to the adjacent fibroblasts, and to act as a specific activator of immature MMP-2 (also called proMMP-2) (Sato et al., 1994). The MT1-MMP/MMP-2 system is important for tumor cells to invade the basement membrane by degrading type IV collagen, a preferred MMP-2 substrate, and then the stroma by degrading type I collagen, a preferred MT1-MMP substrate (Stetler-Stevenson et al., 1993). To activate proMMP-2 on the cell surface, at least two molecules of MT1-MMP must be in close proximity. This is achieved by the formation of a homophilic MT1-MMP complex through interactions between their haemopexin-like domains (see Fig. 1.10, Introduction). One of the enzymes in the complex binds to TIMP-2 to form an enzyme-inhibitor complex. The exposed TIMP-2 C-terminal domain then binds to the proMMP-2 haemopexin-like domain, positioning proMMP-2 optimally for activation by the other active MT1-MMP molecule. This activation is thought to be a two-step process: the first one, involving MT1-MMP catalytic activity, generates the intermediate form (64 kDa), and the second one, due to an intermolecular autocatalytic reaction or to extracellular factors (e.g. plasmin), results in the formation to the mature form (62 kDa) (Baramova et al., 1997).

High levels of MT1-MMP expression and MMP-2 activation in tumors correlate with a highly invasive phenotype. However, carcinoma cells in tissue and cancer cell lines, such as A375 melanoma cells, rarely express MMP-2 at high levels, even though they can activate MMP-2 derived from the surrounding fibroblasts by using cell-surface MT1-MMP. This implies that, unless one uses

co-culture systems, experimental conditions can be very different depending on MMP levels expressed by the cells analyzed. HT1080 fibrosarcoma cells are a good model cell system to study the regulation of MT1-MMP-dependent MMP-2 activation because they express high levels of both MT1-MMP and MMP-2.

In this context, I decided to understand the role of different MT1-MMP forms on activation of MMP-2 by promoting or inhibiting MT1-MMP processing and activity in HT1080 fibrosarcoma cells. MT1-MMP overexpression on the cell surface of HT1080 promotes activation of proMMP-2 *in vitro* and enhances cellular invasion of the reconstituted basement membrane (Sato et al., 1994). For this purpose, I set up and used gelatin zymographic methods. These are designed to analyze the proteolytic capacity of latent and active soluble MMPs (Heussen and Dowdle, 1980), as described in Materials and Methods.

3.2.6.2 Effect of MT1-MMP overexpression on MMP-2 activation in HT1080 fibrosarcoma cells

Fig. 3.13 A shows an immunoprecipitation assay from MT1-MMP-transfected HT1080 cells, pulse-labelled with [^{35}S]methionine/cysteine for 2h. Upon these experimental conditions, the total amount of the 60 kDa form of MT1-MMP is reduced compared to non-transfected HT1080 cells, and the 63 kDa form is accumulated in both Triton X-100-soluble (S) and insoluble (P) membrane fraction.

This evidence was unexpected because I have verified that, although basal degradation of gelatin is somewhat increased, MT1-MMP overexpression nevertheless confers a destructive gelatin degradation phenotype in HT1080 similarly to A375 cells (Fig. 3.14). In addition, previous data in MT1-MMP-transfected A375 cells showed that gelatin degradation is only due the 60 kDa form (see Fig. 3.10). On the other hand, HT1080 fibrosarcoma cells do show different biological features from A375 melanoma cells; in HT1080, the basal expression of MT1-MMP and MMP-2 is relatively higher. Hence, a possible

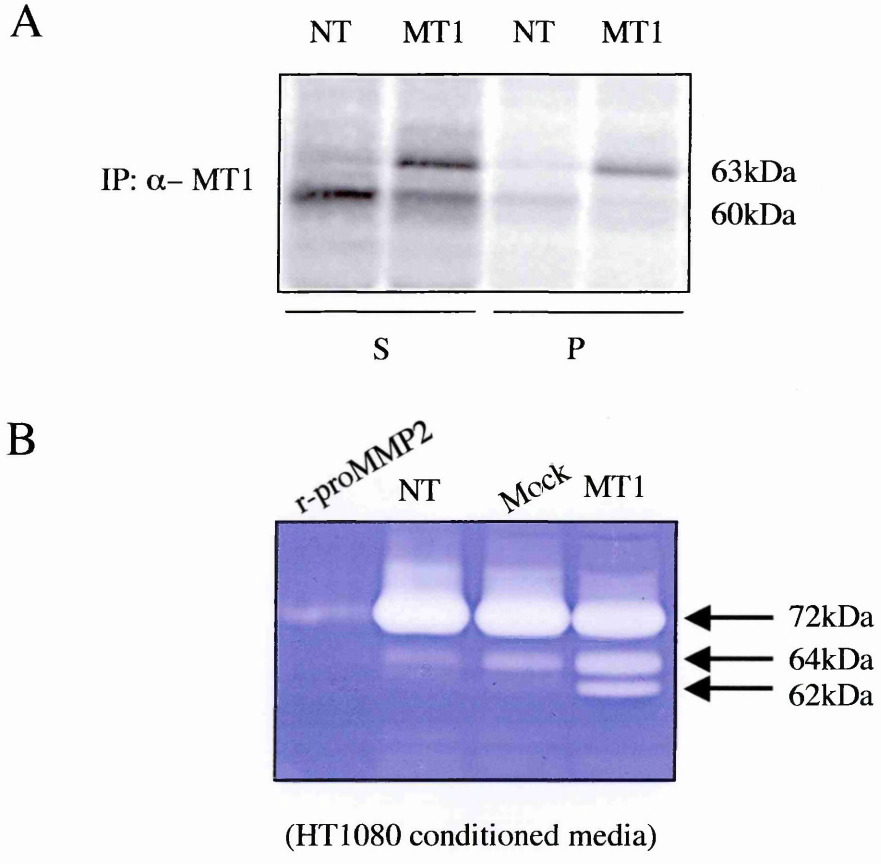


Fig. 3.13. Effect of MT1-MMP overexpression in HT1080 fibrosarcoma cells on MT1-MMP processing and MMP2 activation. (A) Non-transfected (NT) and MT1-MMP-trasfected (MT1) HT1080 cells were pulsed for 2 hours with [^{35}S]methionine/cysteine and incubated with 1% Triton-X-100 at 4°C. Soluble (S) and insoluble (P) fractions were separated by ultracentrifugation, immunoprecipitated with MMR2 antibody and analyzed by SDS-PAGE, as described in Materials and Methods. (B) ProMMP2 activation analysis of conditioned media from non-transfected (NT), pcDNA3.0-transfected (Mock) and MT1-MMP-transfected (MT1) HT1080 cells. A small amount of recombinant proMMP2 (purchased from Sigma-Aldrich, WI, USA) was also loaded as a molecular weight marker (r-proMMP2). Zymogram analysis was performed as described in Materials and Methods. Samples were resuspended in sample buffer without reducing agent and applied without boiling to 10% SDS-PAGE gels containing 1 mg/ml gelatin. The black arrows mark the positions of the MMP2 forms detected: calculated molecular weights are, respectively, 72, 64 and 62 kDa.

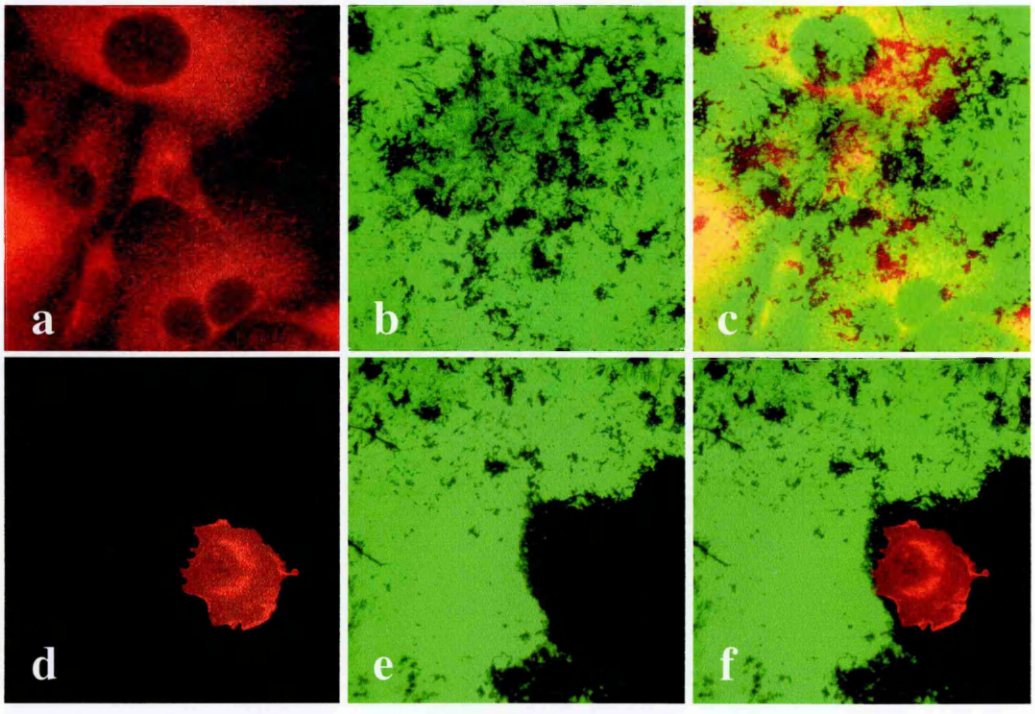


Fig. 3.14. MT1-MMP overexpression in HT1080 fibrosarcoma cells is responsible for a widespread gelatin degradation. Non-transfected (a) and MT1-MMP-transfected (d) HT1080 fibrosarcoma cells were cultured on coverslips coated with FITC-labeled gelatin (b, e). After 16 h of incubation, cells were fixed, labeled with polyclonal immunopurified anti-MT1-MMP antibody MMR2 and visualized by wide-field fluorescence microscopy. A merge section is also shown (c, f).

explanation for this unexpected phenotype could be the contribution of active MMP-2 to gelatin degradation. In fact, when I performed a zymographic analysis from MT1-MMP-transfected HT1080 conditioned media, I found that a very high fraction of MMP-2 is present in its 62 kDa active form (Fig. 3. 13 B).

The possible interpretation of this set of data is that, in HT1080 fibrosarcoma cells, MT1-MMP overexpression is responsible for an unexpected accumulation of its immature 63 kDa form in different membrane fractions. This accumulation might be due to the saturation of the molecular machineries involved with MT1-MMP processing and seems to be correlated with a stronger gelatinolytic activity as well as higher MMP-2 activation levels.

3.2.6.3 Effect of furin inhibitor α_1-PDX and catalitically inactive MT1-MMP mutant (E240A) on MMP-2 activation in MT1-MMP overexpressing HT1080 cells

As discussed above, the accumulation of proMT1-MMP in HT1080 is associated with increased MMP-2 activation. To understand how this activation is related to the catalytic activities of MT1-MMP, I analysed activation pattern of MMP-2 from HT1080 cells co-transfected with MT1-MMP and α1-PDX. Fig. 3.15 shows that α1-PDX overexpression interferes only with the second step of proMMP-2 activation: the conversion of the intermediate 64 kDa form, generated upon proMT1-MMP accumulation, into a 62 kDa form. This result is at variance with previously published evidence that in HT1080 cells MT1-MMP catalytic activity involved only in the first step of proMMP-2 activation, while the second step relies on the extracellular plasminogen activator/plasmin system (Baramova et al., 1997). Of note, a similar phenotype was also produced when I overexpressed the catalytically inactive MT1-MMP mutant (E240A): proMMP-2 was converted to its intermediate 64 kDa form without a significant maturation to its 62 kDa form (Fig. 3.15).

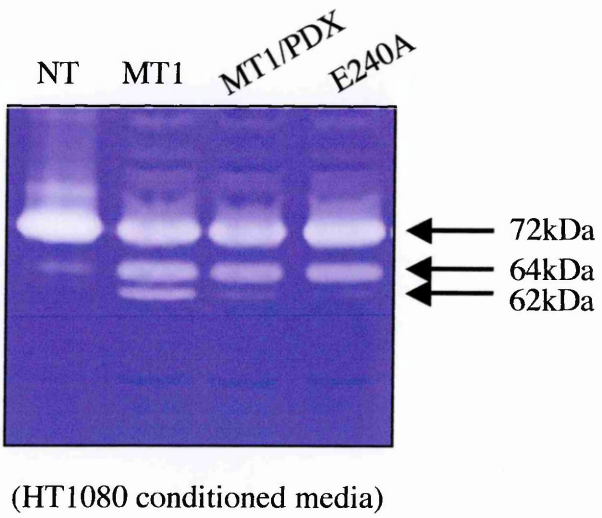


Fig. 3.15. Molecular characterization of MMP2 processing in HT1080 fibrosarcoma cells. Media conditioned by HT1080 cells transfected with MT1-MMP (MT1), cotransfected with MT1-MMP and α1-PDX (MT1/PDX) or catalitically inactive MT1-MMP mutant (E240A) were collected and concentrated by centrifugation. Samples were resuspended in sample buffer without reducing agent and applied without boiling to 10% SDS-PAGE gels containing 1 mg/ml gelatin. The black arrows mark the positions of the MMP2 forms detected: calculated molecular weights are, respectively, 72, 64 and 62 kDa.

As reported above, plasma membrane proMMP-2 activation is generally thought to be a two-step process: the first one, involving MT1-MMP catalytic activity, generates the intermediate form (64 kDa), and the second one, due to an intermolecular autocatalytic reaction or to extracellular factors (e.g. plasmin), results in the formation of mature MMP-2 (Baramova et al., 1997).

I have shown here that only this second step seems to be partially inhibited when the catalitically inactive mutant (E240A) is overexpressed and fully blocked when MT1-MMP is cotransfected with α1-PDX. Taken together, these results suggest that the immature MT1-MMP form (63 kDa) would promote the first step of MMP-2 activation and the catalytically active MT1-MMP form (60 kDa) would be required in the second one.

3.3 Discussion

3.3.1 Molecular and functional significance of MT1-MMP processing depending on its membrane partitioning

In MT1-MMP-transfected A375 melanoma cells, the majority of MT1-MMP is associated with DRM in accord with previous reports (Annabi et al., 2001; Annabi et al., 2004). In contrast, it was recently reported that a MT1-MMP deletion mutant lacking the cytosolic C-terminal tail, but not the full-length protein, is stably partitioned into DRM (Rozanov et al., 2004a). I extended these findings by analyzing the relationship between proteolytic processing and membrane subdomain association. I found that MT1-MMP undergoes proteolytic processing only if associated with detergent-soluble membranes. The mature 60 kDa form is catalytically active, directly involved with gelatin degradation and rapidly processed. On the other hand, the immature 63 kDa form remains stably associated with DRM-enriched membranes and persists following the processing of the detergent-soluble proMT1-MMP and its degradation. In other words, the

metabolic fate and thus the activity of MT1-MMP is directly dependent on its differential association with the two membrane subdomains. A first consequence is that two cellular and possibly functionally distinct pools of MT1-MMP can thus be defined: one that is normally and completely processed and thus available for ECM degradation and/or other proteolytic activities, a second, whose function remains to be better understood, that is however potentially available at the plasma membrane for interactions with other molecules. A second consequence is that this phenomenon, if undetected (depending on the experimental procedures used), could have generated some discrepancies in the interpretation of MT1-MMP processing. The biochemical analysis of MT1-MMP processing was mainly performed from total cell lysates (Cao et al., 1996; Yana and Weiss, 2000): detection of mature MT1-MMP is experimentally not easy depending on both the antibodies used and the preparation of cell lysates (see Fig. 3.1 A).

Most recently MT1-MMP was found in DRM and thus suggested to be associated with caveolin-1 (Annabi et al., 2001); also, it was recently hypothesized that MT1-MMP is internalized via caveolae in addition to the clathrin-mediated pathway (Remacle et al., 2003). In my experiments, I did not find morphological evidence of MT1-MMP association to caveolae-like domains (see Chapter 4). This is not incongruous since caveolae represent only a subpopulation of the total DRM-fraction and can actually be separately isolated from them (Schnitzer et al., 1995).

3.3.2 Characterization of MT1-MMP association with detergent-resistant membranes

In contrast to GPI-anchored proteins, which use their GPI anchor to associate with lipid rafts, the ability of the MT1-MMP molecule to promote the formation of "lipid-enriched" DRM might be not only encoded in protein structure but might also be a consequence of protein oligomerization.

Interestingly, sequence alignment analysis of MT1-MMP resulted in close matches to aromatic-rich consensus caveolin-binding motifs (FXFXXXF, FXXXXFXXF or FXFXXXXFXXF, where F represents Trp, Phe or Tyr), reported to interact with the caveolin-scaffolding domain (Couet et al., 1997). Four of these potential sequences were found in several members of the MMP family and were all located in their haemopexin-like domain, a region known to have a functional role in substrate binding and/or in interactions with TIMPs (Massova et al., 1998). The most conserved caveolin-binding motif found in MT1-MMP was found in its haemopexin-like domain and was located between Tyr323 and Phe333 (YFFRGNKYYRF), whereas the three other less well conserved sequences were also found in the haemopexin-like domain and were located respectively between Phe229 and Phe236, Phe277 and Phe285, and Tyr371 and Phe381. Interestingly, all of the four caveolin-binding motifs were located at each of the putative antiparallel four-stranded β sheets within the hydrophobic core of the proposed topology of the haemopexin-like domain of MMPs (Gohlke et al., 1996). The most likely candidate for membrane targeting is thus the haemopexin-like region of MT1-MMP close to the membrane which might allow partitioning into a lipid-rich environment, either by its physical properties by binding directly to a raft structural protein (Scheiffele et al., 1997).

Sorting determinants in MT1-MMP membrane partitioning could also reside in the cytoplasmic domain, membrane anchor or other extracellular domains. Cytoplasmic domain determinants include the basolateral-targeting, tyrosine-based and dileucine motifs (Matter et al., 1994), PDZ-domain-binding motifs and a growing list of unrelated sequences (Altschuler et al., 2003; Muth and Caplan, 2003). Of note, Strongin and coworkers analyzed a mutant MT1-MMP consisting of a deletion of the cytosolic domain, described to be essential for endocytic routes by binding adaptor proteins (Rozanov et al., 2004a): this mutant protein was still found to be highly enriched in DRM. This evidence would imply the role of intracellular adaptor proteins in regulating MT1-MMP

membrane partitioning.

Determinants in membrane partitioning also include the transmembrane domains of some apically targeted viral proteins (Kundu et al., 1996) and GPI anchors. The latter usually confer localization to the apical membrane (Lisanti et al., 1989) but alone are not always sufficient (Brown and London, 2000). Both *N*- and *O*-glycosylation of the extracellular domain have been implicated in apical targeting (Scheiffele et al., 1997).

From this discussion, it might be interesting not only to investigate, by using specific inhibitors, whether *N*- and *O*-glycans are involved in regulating DRM-association of MT1-MMP as well as its intracellular trafficking and processing but also whether the transmembrane domain or the cytosolic tail are somehow involved in MT1-MMP membrane partitioning.

3.3.3 DRM-associated proMT1-MMP

At steady state, I observed transfected proMT1-MMP to be mainly associated with DRM, with a significant fraction residing at the plasma membrane. This suggests that: 1) intracellular removal of the propeptide domain is not a prerequisite for MT1-MMP cell surface presentation; 2) DRM-associated proMT1-MMP is not subject to intracellular furin-dependent activation and 3) proMT1-MMP is not directly functioning as a gelatinase. A possible mechanicistical explanation is provided by my observation that furin is excluded from detergent-resistant membranes. In fact, physical interaction between proteins associated with different membrane subdomains is extremely unlikely, as recently suggested for other proteins (Ehehalt et al., 2003). In this context, colocalization of furin and MT1-MMP in common structures (see Chapter 4), supports the idea that detergent soluble and resistant MT1-MMP pools might coexist albeit not necessarily interact. This suggests that partitioning of MT1-MMP into different lipid subdomains could be a mechanism for the regulation of its processing and activity similarly to the Alzheimer precursor protein (Ehehalt et al., 2003).

In this chapter, I reported several experiments performed in HT1080 fibrosarcoma cells revealing the differential role of immature and mature MT1-MMP forms in MMP-2 activation. Although it is not definitively demonstrated, I could speculate that the DRM-associated pool of MT1-MMP is somehow responsible for the regulation of MMP-2 activation and is involved in the stable presentation of proMMP-2 at the plasma membrane. DRM-associated proMT1-MMP could also be a reservoir of yet to be defined activities (e.g signaling events, MMP-2 activation). Interestingly, it was recently proposed that the cholesterol content of tumor cells is critical for the regulation of MT1-MMP cell surface presentation and MMP-2 activation (Atkinson et al., 2004). Again, although the role of DRM-associated proMT1-MMP is still obscure, this pool could be potentially available for extracellular interactions, since it seems to be stably expressed over the plasma membrane. Of note, lipid raft domains represent a preferred platform for a number of signaling receptors; hence, DRM-associated proMT1-MMP pool at the plasma membrane could be involved in signalling-related functions. A recent paper has indirectly identified a role for MT1-MMP, overexpressed in different cell lines, in the regulation of cell proliferation by affecting cyclin-dependent kinases (Hotary et al., 2003); this event could be in line with my proposed hypothesis that will be discussed in-depth in Chapter 5.

CHAPTER 4

Functional and Morphological Characterization of the Intracellular Activation Compartment of MT1-MMP

4.1 Introduction

The activation of proMT1-MMP has been under intense debate. Some reports have suggested that proMT1-MMP has to be processed to activate MMP2 (Cao et al., 1998; Deryugina et al., 2004; Pei and Weiss, 1996; Yana and Weiss, 2000; Yu et al., 1997). At variance with this, others have reported that the full-length proMT1-MMP could act as an active enzyme. Moreover, several groups have also found proMT1-MMP at the plasma membrane of different cell lines, adding weight to the proposition that the propeptide domain of MT1-MMP is required for the efficient trafficking of the enzyme to the cell surface (Cao et al., 2000; Sato et al., 1994; Sternlicht and Werb, 2001), and that MT1-MMP activation would occur on the plasma membrane (Mayer et al., 2003). Still, the exact site of MT1-MMP activation remains obscure.

By investigating MT1-MMP processing and activation, I have found that the association of MT1-MMP with different membrane subdomains establishes divergent metabolic fates and might be crucial in the control of its different activities. In particular, I have shown that only the detergent-soluble fraction of MT1-MMP undergoes processing to the mature form in a furin-dependent manner. Only the mature form is catalytically active and directly responsible for ECM degradation. These data also demonstrate that the vast majority of MT1-MMP is indeed localized to the cell surface in its precursor form. This means that,

although the propeptide domain does not interfere with MT1-MMP trafficking to the cell surface, it might have to be cleaved to allow activation.

Of note, recent data also show that furin and proMT1-MMP colocalize in the Golgi apparatus, in intracellular post-Golgi/endosomal structures as well as at the plasma membrane (Mayer et al., 2003). Thus, furin appears to be associated with MT1-MMP in the secretory pathway independently of the cleavage of the MT1-MMP propeptide domain. Indeed, this was directly confirmed for proMT3-MMP in MDCK cells (Kang et al., 2002). These results imply that, besides pro-domain processing, furin could be involved in the trafficking of proproteins to the cell surface.

The definition of the MT1-MMP activation compartment and the characterization of the possible, not strictly enzymatic, interaction between furin and proMT1-MMP are the subjects of this chapter.

4.2 Results

4.2.1 Analysis of the subcellular localization of MT1-MMP

As mentioned above, the MT1-MMP processing compartment still needs to be defined. To address this issue, I first analyzed by indirect immunofluorescence light microscopy the subcellular distribution of MT1-MMP at steady state in A375 melanoma cells by using the immuno-purified polyclonal anti-MT1-MMP antibody (MMR2): MT1-MMP presented a perinuclear patchy distribution with scattered cytoplasmic elements and plasma membrane staining (Fig. 4.1). Next, I compared the localization of MT1-MMP to markers of the secretory pathway. MT1-MMP showed no significant colocalization with endoplasmic reticulum (PDI, protein disulfide isomerase) and Golgi apparatus (Giantin) (4.1 C and F). Instead, when I tested an anti-TGN46 antibody in MT1-MMP-transfected A375 cells, a patchy asymmetrical perinuclear distribution was

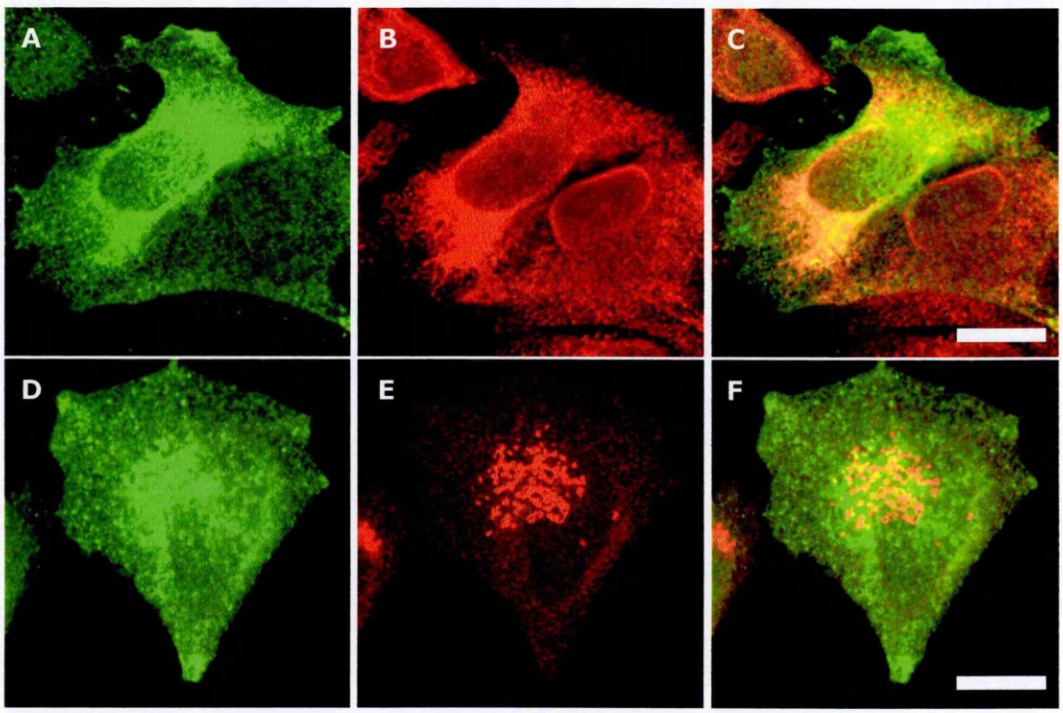


Fig. 4.1. Subcellular distribution of MT1-MMP in A375 melanoma cells by immunofluorescence (I). A375 cells transfected with MT1-MMP were plated on coverlips for 16 hours, then fixed and analyzed at the confocal microscope as described in Material and Methods section. Cells were double-labeled with immunopurified polyclonal anti-MT1-MMP antibody MMR2 (A, D) and with monoclonal antibodies respectively directed against PDI (B), Giantin (E). Merged staining is also shown on the right (C, F). Scale bars: 10 μm.

observed, with a high, albeit not complete, colocalization with MT1-MMP (Fig. 4.2 C); TGN46 is a protein known to cycle between the trans-Golgi network, the plasma membrane and the endosomal compartment, and although generally considered to be a trans-Golgi network marker, its actual distribution depends on the cell type (for a review, see Keller and Simons, 1997). In contrast, as bona fide early and late endosome markers, I tested the distribution of transferrin receptor (a plasma membrane-endosome recycling protein) and mannose-6-phosphate receptor (a TGN-endosome recycling protein) respectively, and found them to be only partially overlapping with MT1-MMP (Fig. 4.2 F and I, respectively).

In which of these compartments, therefore, does furin-dependent activation actually occur? My pulse-chase analysis showed that fully processed MT1-MMP appeared only after at least 20 minutes, suggesting that it might occur in a late station along the biosynthetic-secretory pathway (see Fig. 3.6). In addition, a furin-dependent proteolytic activation is not expected to occur before the trans-Golgi network (Molloy et al., 1999). Still, to formally rule out the possibility that MT1-MMP activation occurs in an early station of the secretory pathway, I took advantage of the properties of the fungal toxin Brefeldin A (BFA). Treatment with BFA leads to intermixing between the endoplasmic reticulum and the Golgi apparatus and blocks exit of a secretory cargo from the Golgi apparatus itself (Fujiwara et al., 1988; Lippincott-Schwartz et al., 1989). Upon BFA treatment, proteins normally present in the endoplasmic reticulum and the Golgi apparatus would still be modified by any processing enzymes contained in those compartments: even so, I observed no proteolytic processing of proMT1-MMP (Fig. 4.3 A); moreover its cell surface presentation is strongly reduced (Fig. 4.3 B). Interestingly, the intracellular MT1-MMP pool does not follow the fate of the rest of the Golgi fusing into the ER upon short BFA treatment (Fig. 4.3 C), although it appears to be more tubulated if compared with the normal profile (see Fig. 4.1 D-F). Given that some intracellular compartments becomes more tubulated but resistant to BFA (TGN, lysosomes,

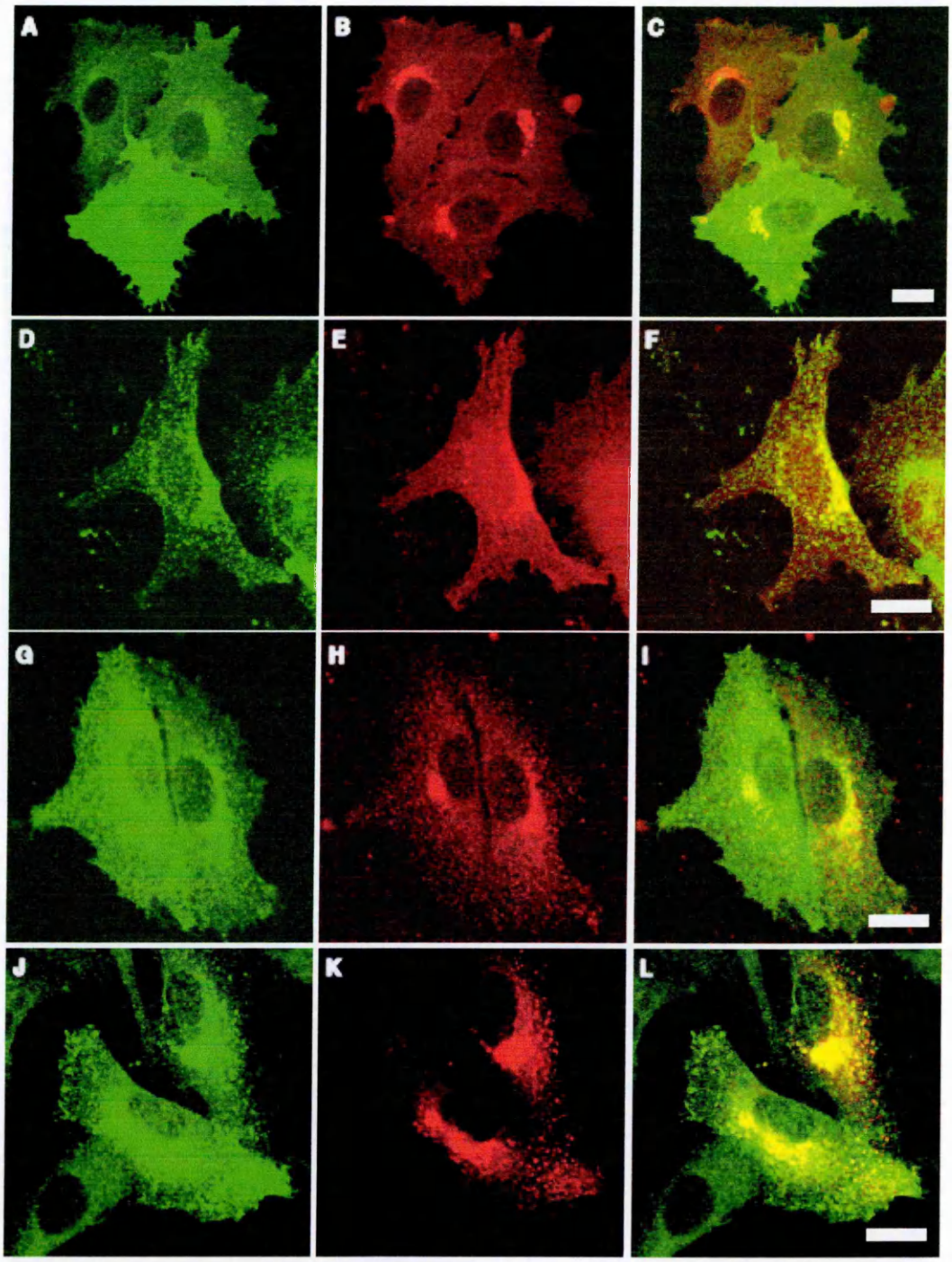


Fig. 4.2. Subcellular distribution of MT1-MMP in A375 melanoma cells by immunofluorescence (II). A375 cells transfected with MT1-MMP and pcDNA3.0 (A-I) or MT1-MMP and Furin-GFP (J-L) were plated on coverlips for 16 hours, then fixed and analyzed at the confocal microscope. Cells were double-labeled with immunopurified polyclonal anti-MT1-MMP antibody MMR2 (A, D, G, J) and with antibodies directed against TGN46 (B), transferrin receptor (E) and mannose 6-phosphate receptor (H). Furin-GFP staining is shown in red (K). Merged staining is also shown (C, F, I, L). Scale bars: 10 μm.

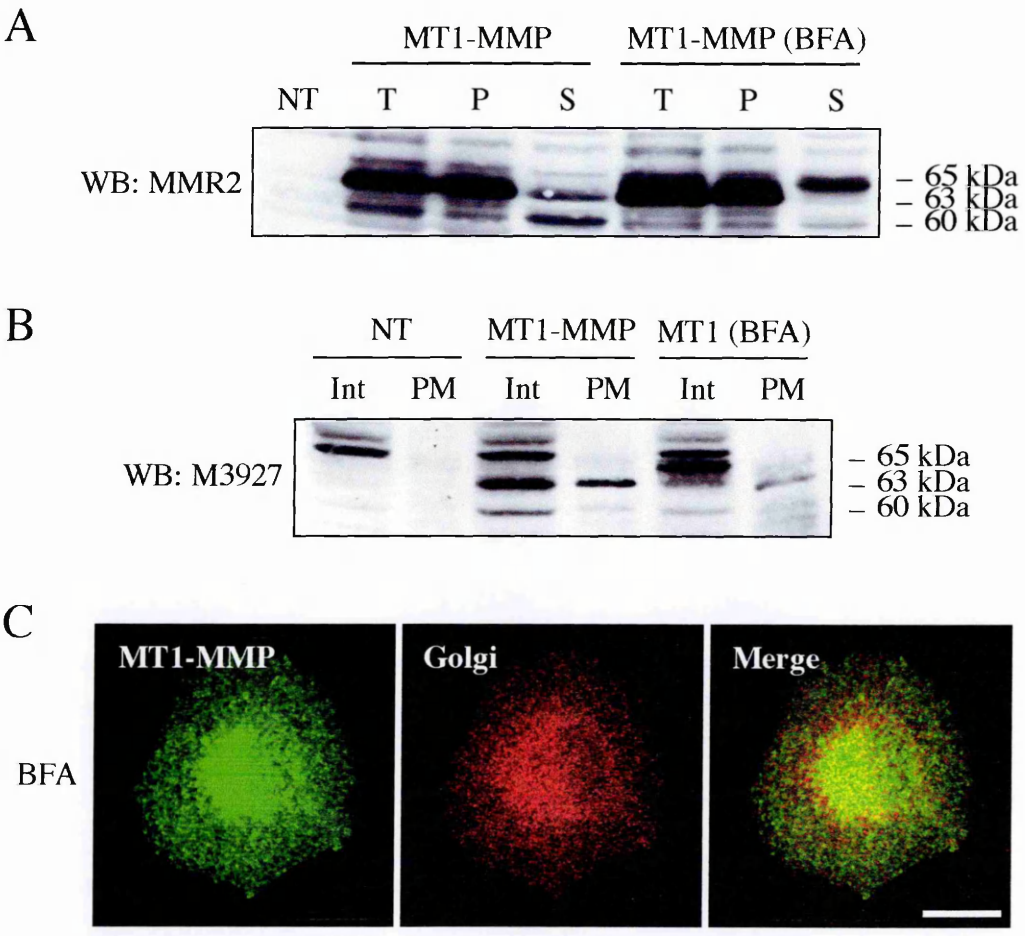


Fig. 4.3. Effect of BFA on MT1-MMP processing, cell surface presentation and intracellular distribution. (A) Untransfected (NT) and MT1-MMP-transfected A375 cells were lysed with 1% Triton X-100-containing TNE buffer, as described in Materials and Methods. Insoluble (P) and soluble (S) fractions were separated from total lysates (T) by ultracentrifugation at 4°C (120,000 x g, 1 hour), resuspended in sample buffer and subjected to SDS-PAGE, transferred to nitrocellulose membrane and visualized by Western blotting with the MMR2 antibody. (B) Untransfected (NT) and MT1-MMP-transfected A375 cells were biotinylated at 4°C and then lysed with a TNE containing 1% Triton X-100, as described in Materials and Methods. After centrifugation at 4°C (13,000 x g, 10 minutes), intracellular proteins (Int) were separated from biotinylated plasma membrane proteins (PM) by capture with streptavidine-conjugated agarose beads. Each fraction was subjected to SDS-PAGE and transferred on nitrocellulose membrane. The MT1-MMP distribution profile was analyzed with polyclonal anti-MT1-MMP antibody M3927. The 65, 63 and 60 kDa forms are indicated on the right. Where indicated, cells were treated with BFA (10 μg/ml) for 2 hours prior to processing. (C) MT1-MMP-transfected A375 cells, treated with BFA for 15 min, were double-labeled with immunopurified polyclonal anti-MT1-MMP antibody MMR2 (MT1-MMP) and with monoclonal antibody directed against a Golgi marker (Giantin). Merged staining is also shown on the right. Scale bar: 10 μm.

early and recycling endosomes) (Lippincott-Schwartz et al., 1991), it seems clear that, at steady-state, MT1-MMP is concentrated in a TGN/post-TGN compartment, where its furin is highly concentrated.

To further determine whether furin activates proMT1-MMP in the trans-Golgi network, I investigated the subcellular localization of furin and MT1-MMP in more detail by confocal microscopy. Furin is, depending on the cell type, typically expressed at very low levels and distributed throughout the various proprotein processing compartments including the trans-Golgi network, cell surface and endosomes (Mayer et al., 2004b; Molloy et al., 1999). I thus cotransfected A375 cells with MT1-MMP and a furin-GFP chimera. The double-labeled cells were subsequently sectioned optically with z-series on a Zeiss LSM510 confocal system. Extensive overlap between MT1-MMP and furin was observed in the perinuclear area (Fig. 4.2 L).

4.2.2 MT1-MMP processing takes place in an intracellular, post-trans-Golgi network compartment

Considering this evidence, I further searched for the MT1-MMP activation compartment. The first possible station considered for MT1-MMP processing was thus the trans-Golgi network, a complex structure located at the distal-most region of the Golgi apparatus, which plays a central role in the sorting, processing and targeting of proteins of the secretory pathway en route to their final destination. When MT1-MMP was accumulated in the trans-Golgi network through the application of a 20°C temperature block (Griffiths et al., 1985) (Fig. 4.4 A), it remained unprocessed (Fig. 4.4 B). Within 30 minutes after temperature shift to 37°C (release from the block), intracellular trafficking as well as MT1-MMP processing resumed (Fig. 4.4 B). Hence, my results are consistent with a MT1-MMP processing event occurring after exit from the trans-Golgi network. It should be mentioned previous reports have shown that, in these conditions, the

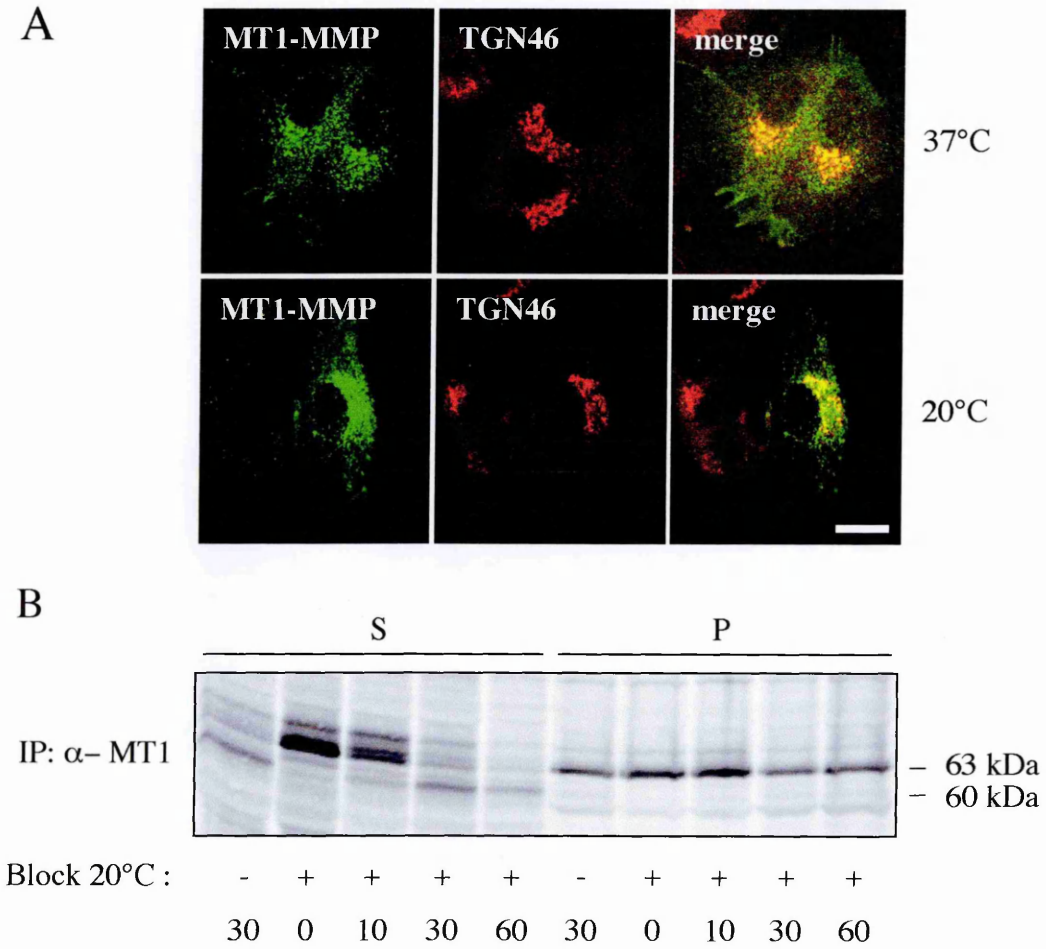


Fig. 4.4. Effect of 20°C block on MT1-MMP processing in A375 melanoma cells. (A) A375 cells transfected with MT1-MMP were plated on coverlips for 16 hours, incubated at 20°C for 30 min, where indicated, and then fixed and analyzed at the confocal microscope. Cells were double-labeled with immunopurified polyclonal anti-MT1-MMP antibody MMR2 (MT1-MMP) and with polyclonal sheep antibody directed against TGN46 (TGN46), Merged staining is also shown. Scale bars: 20 μm. (B) MT1-MMP-transfected A375 cells were pulse-labeled with [^{35}S]methionine/cysteine for 5 minutes. Labeled proteins were accumulated in the trans-Golgi network by incubating the cells at 20°C for 30 minutes, then released at 37°C for different lengths of time, as indicated. Triton X-100-soluble (S) and insoluble (P) fractions were separated by ultracentrifugation, immunoprecipitated and analyzed by SDS-PAGE/autoradiography.

enzymic activity of furin is not blocked (Song and Fricker, 1995). Considering all the above and that proteins blocked in the trans-Golgi network at 20°C are known to exit rapidly (Polishchuk et al., 2000), I suggest that the processing of MT1-MMP takes place beyond the trans-Golgi network during transport to the plasma membrane.

Next, I determined whether processing of MT1-MMP takes place prior to, or after, delivery to the plasma membrane. Tannic acid has been used as an inactivator of fusion events at the plasma membrane (Newman et al., 1996; Polishchuk et al., 2004). I thus proceeded to use this experimental approach to prevent delivery of MT1-MMP to the cell surface after a 20°C temperature block. In the presence of tannic acid, proMT1-MMP processing still occurred albeit not completely (Fig. 4.5 A). Even though some toxic effects of the treatment can be inferred from the increased degradation of MT1-MMP, this is taken only to indicate that MT1-MMP processing can occur intracellularly in a post-trans-Golgi network compartment and prior to arrival at the plasma membrane. To validate that tannic acid indeed functioned to block fusion at the plasma membrane in our experimental system, the delivery of a temperature sensitive mutant (ts045) of vesicular stomatitus virus G protein (VSV-G)-GFP chimera to the plasma membrane was verified. This protein is blocked in the endoplasmic reticulum while cells are maintained at 40°C, is regularly transported through the Golgi apparatus up to the TGN (where it remains) at 20°C and is finally transported to plasma membrane at the permissive temperature of 32°C. As shown in Fig. 4.4 B, control cells feature a clearly defined plasma membrane staining 60 minutes after release from the 20°C block; cells treated with 0.5% tannic acid, instead, are unable to deliver (VSV-G)-GFP to the cell surface. Thus, in A375 cells treated with tannic acid, fusion of transport intermediates with the plasma membrane is in fact impaired (see graph in Fig. 4.5 B).

The extracellular face of the plasma membrane has been recently suggested to be the location where MT1-MMP processing by furin might occur

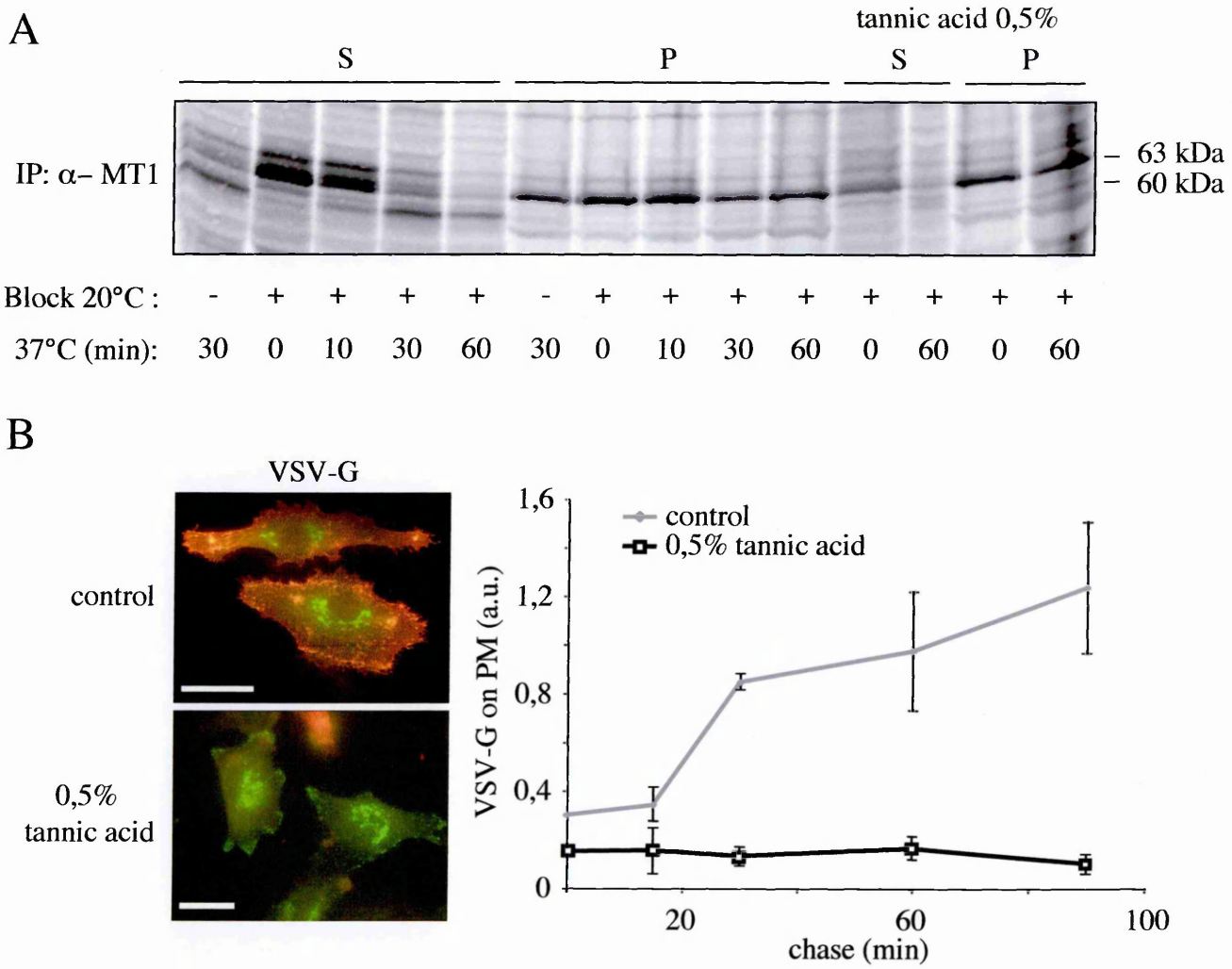


Fig. 4.5. MT1-MMP processing takes place in an intracellular, post-Golgi compartment. (A) MT1-MMP-transfected A375 cells were pulse-labeled with [^{35}S]methionine/cysteine for 5 minutes. Labeled proteins were accumulated in the trans-Golgi network by incubating the cells at 20°C for 30 minutes, then released at 37°C for different lengths of time, as indicated. Triton X-100-soluble (S) and insoluble (P) fractions were separated by ultracentrifugation, immunoprecipitated and analyzed by SDS-PAGE/autoradiography. Tannic acid 0.5% was added during the 20°C block, 10 minutes prior to release at 37°C for 60 minutes. (B) Tannic acid completely blocks arrival of proteins to plasma membrane. A375 cells were transfected with the ts045 temperature sensitive mutant of VSV-G-GFP chimera, incubated at 40°C over night, blocked at 20°C for 30 minutes then chased at 32°C for the indicated time in the absence or presence of 0.5% of tannic acid. Images illustrate representative control (top) and treated (bottom) cells. VSV-G staining on PM as detected from non permeabilized cells with an antibody raised against its extracellular domain is shown in red. Scale bar 10 μm. The graph shows ratio of VSV-G on plasma membrane to total VSV-G at indicated chase time. This experiment was performed in collaboration with Massimiliano Baldassarre (Consorzio Mario Negri Sud).

(Mayer et al., 2003). Since my results are at variance with this notion, I further tested this possibility. Furin is a calcium-dependent enzyme (Molloy et al., 1992) and as such it would be completely inhibited in the absence of free (i.e. unchelated) Ca^{2+}. Fig. 4.6 shows that MT1-MMP processing can still occur when extracellular Ca^{2+} is chelated with a supramaximal concentration of EGTA (5 mM). A minor fraction of proMT1-MMP does not appear to mature (see graph in Fig. 4.6), possibly due to a plasma membrane component of processing (Mayer et al., 2003) or leakage of EGTA into the processing compartment via fluid-phase endocytosis.

4.2.3 Ultrastructural features of the MT1-MMP/furin-positive post-trans-Golgi network compartment

To gain a better understanding of the processing compartment, MT1-MMP-transfected A375 cells were processed for cryoimmuno-electron microscopy and immunolabeled with immunopurified anti-MT1-MMP antibody (MMR2). We thus determined the intracellular distribution of labeling densities for MT1-MMP throughout the various compartments of the secretory pathway, identified on the basis of their morphology. The distribution of MT1-MMP was not homogeneous and presented a clear high peak of 76.6% (Table 4.1) in tubulo-saccular structures located between the trans-Golgi network and the plasma membrane as illustrated in Fig. 4.7 (A-C). Of note, MT1-MMP expression levels had no effect on the morphology of the secretory structures (not shown). This compartment, on the basis of our biochemical experiments and on the morphological observation that, at the ultrastructural level, it was not specifically labeled by the TGN marker TGN46 at steady state (Fig. 4.8), was thus defined as a post-trans-Golgi network compartment. This compartment might also contain endosomal elements as suggested by our immunofluorescence studies, consistent with recent reports suggesting a recycling loop for MT1-MMP {Remacle, 2003

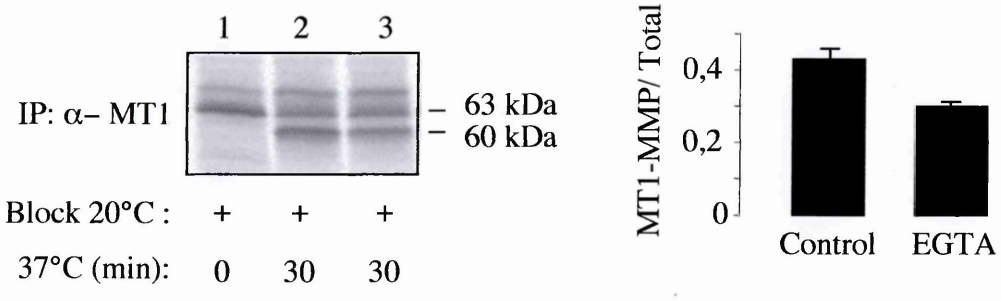


Fig. 4.6. Effect of EGTA on MT1-MMP processing. After incubating the cells at 20°C for 30 minutes, the MT1-MMP-transfected pulsed A375 cells were treated (lane 3) or not (lane 2) with 5 mM EGTA for 30 minutes at 37°C. Only the soluble fraction is shown. MT1-MMP molecular forms in lanes 2 and 3 were quantified with the public domain ImageJ v.1.3 software and plotted on the reported chart as a ratio of MT1-MMP processing.

Table 4.1. Distribution of MT1-MMP over cellular membrane compartments in MT1-MMP-transfected A375 cells

	Gold particles	Labeling density
1. ER	~2.3%	0.04 ± 0.03
2. ER to Golgi intermediate compartments	0.4%	0.07 ± 0.04
3. Golgi cisternae	~1%	0.006 ± 0.01
4. TGN	1.14%	0.017 ± 0.01
5. post-TGN	76.6%	0.87 ± 0.05
6. Plasma membrane	18.5%	0.25 ± 0.07

To determine the distribution of MT1-MMP throughout the secretory pathway in transfected A375 cells, quantification was performed as described in Materials and Methods. Labeling densities are expressed as the number of gold particles per intersections of the morphometrical grid. This quantification was performed by Galina Beznoussenko (Consorzio Mario Negri Sud).

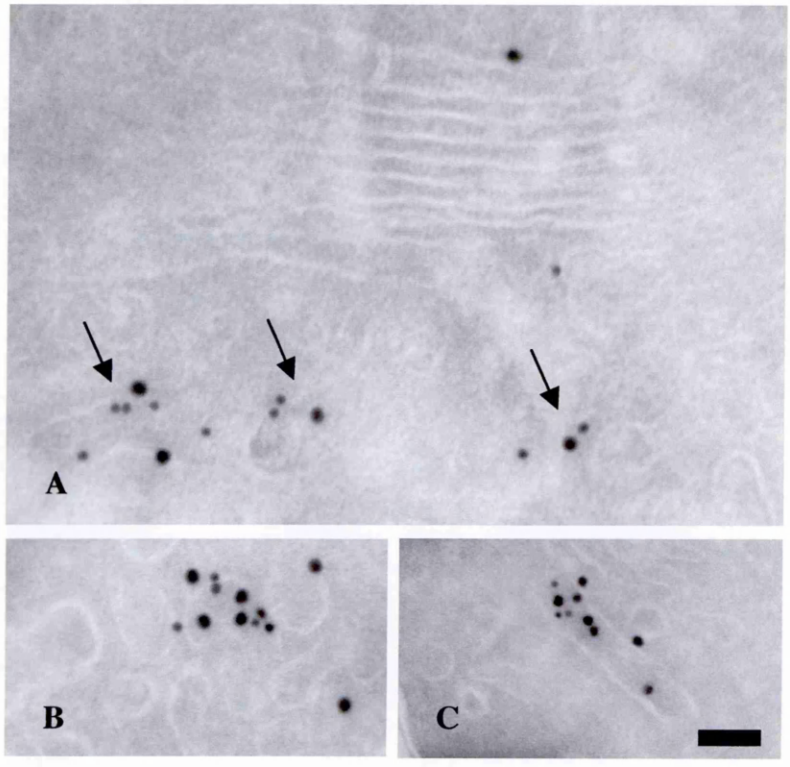


Fig. 4.7. Characterization of a MT1-MMP- and Furin-positive post-Golgi compartment in A375 melanoma cells by cryo-immuno-electron microscopy. MT1-MMP-transfected A375 cells were fixed and prepared for cryo-immunogold labeling as decribed in Materials and Methods by using differently sized gold particles, as indicated. Subcellular colocalization of MT1-MMP with endogenous furin: 10 nm, anti-furin; 15 nm, anti-MT1-MMP (immunopurified antibody MMR2). Arrows mark the positions of specific regions containing both MT1-MMP and furin. Scale bars: 100 nm (A), 75 nm (B, C). Experiment performed in collaboration with Galina Beznoussenko (Consorzio Mario Negri Sud).

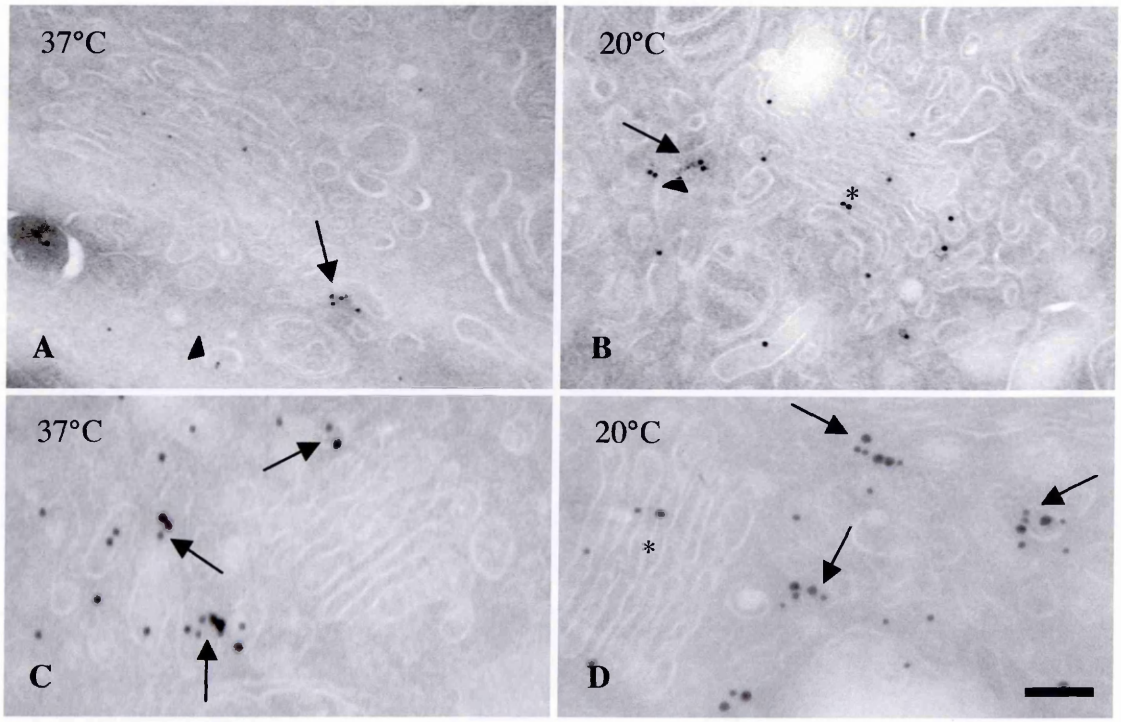


Fig. 4.8. Subcellular colocalization of MT1-MMP with endogenous TGN46 and furin by cryo-immuno electron microscopy. MT1-MMP-transfected A375 cells at steady state (A) or incubated for 30 min at 20°C (B) were fixed and prepared for cryo-immunogold labeling as described in Materials and Methods by using differently sized gold particles, as indicated: at 37°C, 15 nm, anti-TGN46; 10 nm, anti-MT1-MMP (immunopurified antibody MMR2); at 20°C, 5 nm, anti-TGN46; 10 nm, anti-MT1-MMP. Arrows mark the positions of specific areas containing TGN46, arrowheads mark MT1-MMP. Asterisks mark proteins detected at 20°C in Golgi cisternae. Of note in these conditions, MT1-MMP and TGN46 colocalization appears to increase. Scale bar: 200 nm. MT1-MMP-transfected A375 cells at steady state (C) or incubated for 30 min at 20°C (D) were fixed and prepared for cryo-immunogold labeling as described in Materials and Methods by using differently sized gold particles, as indicated: 15 nm, anti-furin; 10 nm, anti-MT1-MMP (immunopurified antibody MMR2). Arrows mark the positions of specific areas containing both furin and MT1-MMP. Asterisks mark proteins detected at 20°C in Golgi cisternae. Scale bar: 150nm. This experiment was performed in collaboration with Galina Beznoussenko (Consorzio Mario Negri Sud).

#269; Wang, 2004 #224}.

When the labeling density of MT1-MMP in different compartments was compared to that of endogenous furin, we established that 64.6% of the MT1-MMP-positive structures contained furin, furthermore 84.8% of the furin-positive structures contained MT1-MMP. This localization coincided with the post-trans-Golgi network compartment, where MT1-MMP was enriched and that also represented the foremost reservoir for endogenous furin in A375 cells (Fig. 4.7, A-C). Of note, the post-TGN compartment where furin and MT1-MMP colocalize, was not significantly affected by a 20°C block (see Fig. 4.8 B).

MT1-MMP had been previously hypothesized to be localized to caveolar DRM (Annabi et al., 2001); it was also recently suggested that MT1-MMP is internalized via caveolae in addition to the clathrin-mediated pathway (Remacle et al., 2003). I thus examined whether the protein caveolin-1, a typical component of caveolae, colocalized with MT1-MMP at the plasma membrane. Fig. 4.9 clearly shows that MT1-MMP at the plasma membrane is not preferentially localized in caveolin-1-enriched domains. This does not conflict with the biochemical data in Fig. 3.4 C showing that both MT1-MMP and caveolin-1 partition into DRM; in fact, caveolae represent only a subset of the total DRM fraction (Schnitzer et al., 1995).

4.2.4 Analysis of the subcellular localization of mature MT1-MMP

I have thus far discussed a set of data suggesting that MT1-MMP processing occurs inside the cell and most likely in association with furin-enriched tubulo-saccular structures located between the trans-Golgi network and the plasma membrane. To better define the subcellular distribution of processed mature MT1-MMP, I made use of a FLAG epitope-tagged MT1-MMP cDNA (defined F112-MT1-MMP for the introduction of the FLAG epitope at the Arg112) (Fig. 4.10 A). Introduction of the FLAG epitope tag at the immediate

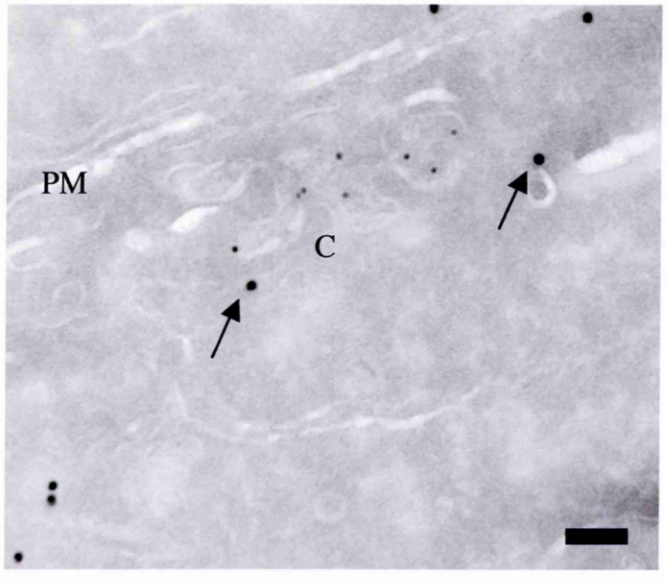


Fig. 4.9. Subcellular and plasma membrane colocalization of MT1-MMP with endogenous caveolin by cryo-immuno-electron microscopy. MT1-MMP-transfected A375 cells were fixed and prepared for cryo-immunogold labeling as decribed in Materials and Methods by using differently sized gold particles, as indicated: 10 nm, anti-caveolin-1; 15 nm, anti-MT1-MMP (immunopurified antibody MMR2). PM indicates plasma membrane rims. C indicates caveosome. Arrows mark the positions of specific regions containing MT1-MMP or caveolin-1. Scale bar: 150 nm. Experiment performed in collaboration with Galina Beznoussenko (Consorzio Mario Negri Sud).

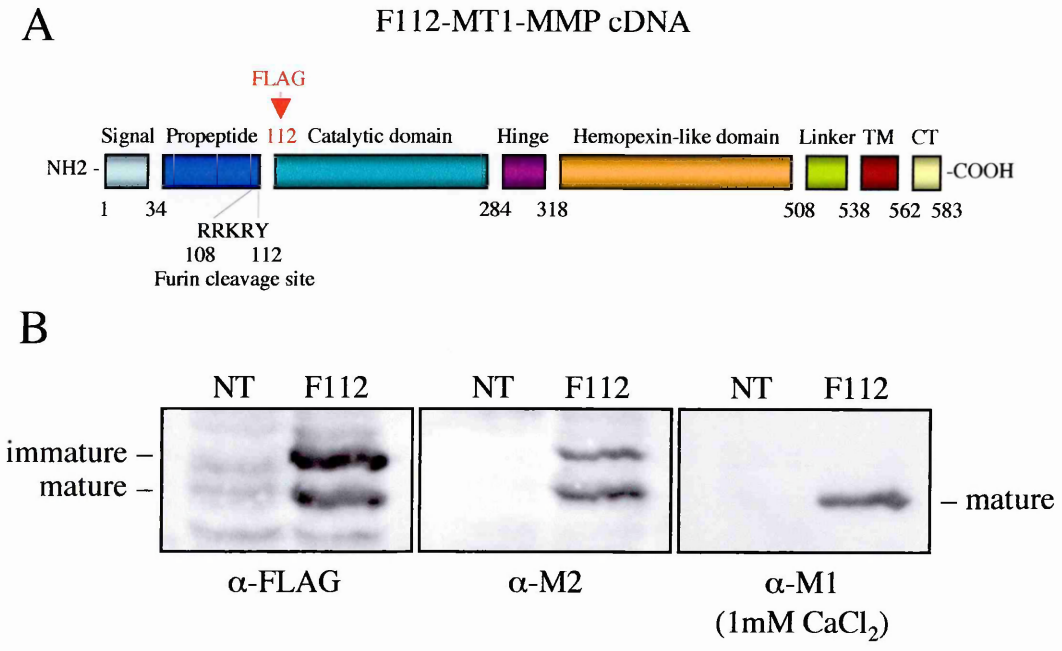


Fig. 4.10. Molecular characterization of the F112-MT1-MMP mutant. (A) Schematic representation of the modular domain structure of MT1-MMP with the insertion of a FLAG epitope (red) between the propeptide and the catalytic domains and soon after the furin binding site (Wu et al., 2004). (B) Untransfected (NT) and F112-transfected A375 melanoma cells were lysed and resuspended in sample buffer and subjected to SDS-PAGE, transferred to nitrocellulose membrane and visualized by Western blotting with different antibodies raised against the FLAG epitope: a) FLAG, an immunopurified polyclonal antibody; b) M2, an immunopurified monoclonal antibody; c) M1, a $CaCl_2$-sensitive immunopurified monoclonal antibody. Different molecular forms of MT1-MMP were detected and respectively defined as immature/mature forms depending on their expected molecular weights.

C-terminal of the propeptide cleavage site permits the use of immunological methods to monitor propeptide cleavage and correlation of this event with enzymic activity. Importantly, the FLAG tag at this position does not significantly affect propeptide cleavage or enzyme activity (Wu et al., 2004). By employing different FLAG peptide-specific antibodies (polyclonal anti-FLAG, mAb M1 and mAb M2), the immature form of MT1-MMP (which reacts with polyclonal anti-FLAG and monoclonal M2 antibodies only) can be distinguished from the mature form (propeptide-less) of MT1-MMP generated by autoproteolytic cleavage C-terminal to Arg112 (Fig. 4.10 B), which reacts also with monoclonal M1 antibody. These results show that the tested antibodies perform as predicted and should therefore serve as highly selective probes for elucidating the intracellular routing and processing of MT1-MMP in cells.

To determine the steady-state location of mature MT1-MMP, A375 cells transfected with MT1-MMP were fixed 12-16 h post-transfection and processed for immunofluorescence microscopy with the different primary antibodies (see Materials and Methods). Under these conditions the staining patterns obtained with M1 and M2 (Fig. 4.11) were apparently quite similar, when compared to the polyclonal anti-FLAG antibody, and consisted of a prominent perinuclear Golgi-like concentration similarly to previous experiments with MMR2 antibody (see Fig. 4.1). In fact, the M1-specific staining was substantially different from that of M2 because only it partially overlapped with the staining pattern obtained with the anti-FLAG antibody. This evidence is in line with the biochemical characterization I performed to define the subcellular compartment where MT1-MMP activation occurs: the mature form of MT1-MMP is processed and mainly present out of the perinuclear Golgi-like area.

To better determine in which compartment mature MT1-MMP was concentrated, a series of colocalization experiments was performed with antibodies directed against a variety of secretory pathway marker proteins. Similarly to the previous analysis performed with the MMR2 antibody,

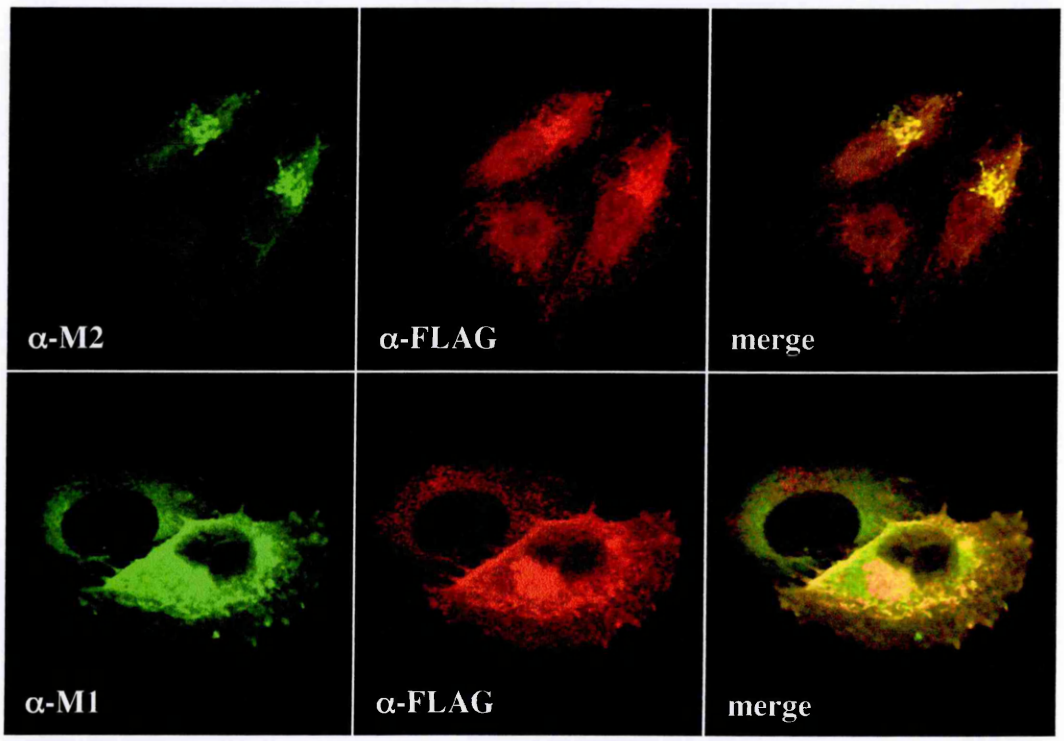


Fig. 4.11. Subcellular distribution of F112-MT1-MMP in A375 melanoma cells by immunofluorescence. A375 cells transfected with F112-MT1-MMP were plated on coverlips for 16 hours, then fixed and analyzed at the confocal microscope as described in Materials and Methods section. To test the anti-M1 antibody, all the solutions were prepared with 1mM $CaCl_2$. Cells were double-labeled with different anti-FLAG antibodies, as indicated. Merged staining is also shown on the right. Scale bar: 10 μm.

the distribution of mature MT1-MMP overlaps extensively with that of TGN46 and furin (Fig. 4.12).

4.2.5 Mature MT1-MMP is associated with intracellular recycling membranes

The colocalization analysis indicates that mature MT1-MMP resides in the post-Golgi area, likely where MT1-MMP processing occurs. This compartment might also contain endosomal elements as suggested by a preliminary morphological analysis I performed with specific markers (see transferrin receptor and mannose-6-phosphate receptor patterns in Fig. 4.2). This would be consistent with recent reports suggesting a recycling loop for MT1-MMP with the plasma membrane (Remacle et al., 2003; Wang et al., 2004b). In these reports, morphological analysis was performed with antibodies that detected both immature and mature MT1-MMP forms. In particular, MT1-MMP was shown to be internalised from the cell surface and to be colocalised with various endocytic compartment markers: EEA1, an early endosomal antigen interacting with Rab5 and phosphatidylinositol-3-phosphate to facilitate early endosome fusion (Christoforidis et al., 1999); Rab4, enriched in recycling endosomes (Sonnichsen et al., 2000), and p230, enriched in the trans-Golgi network (Gleeson et al., 1996). Given these results, it was concluded that MT1-MMP is internalized through the early endosomes and then routed through the trans-Golgi network from where it is recycled back to the cell surface.

Given this context, to better understand the subcellular distribution of mature MT1-MMP within endosomal compartments, I examined whether any of the Rab proteins, Ras-related GTPases known to have a role in endosomal trafficking, colocalized with mature MT1-MMP. Due to the lack of good immunological reagents for the detection of endogenous Rab proteins, I transiently cotransfected A375 cells with F112-MT1-MMP and various

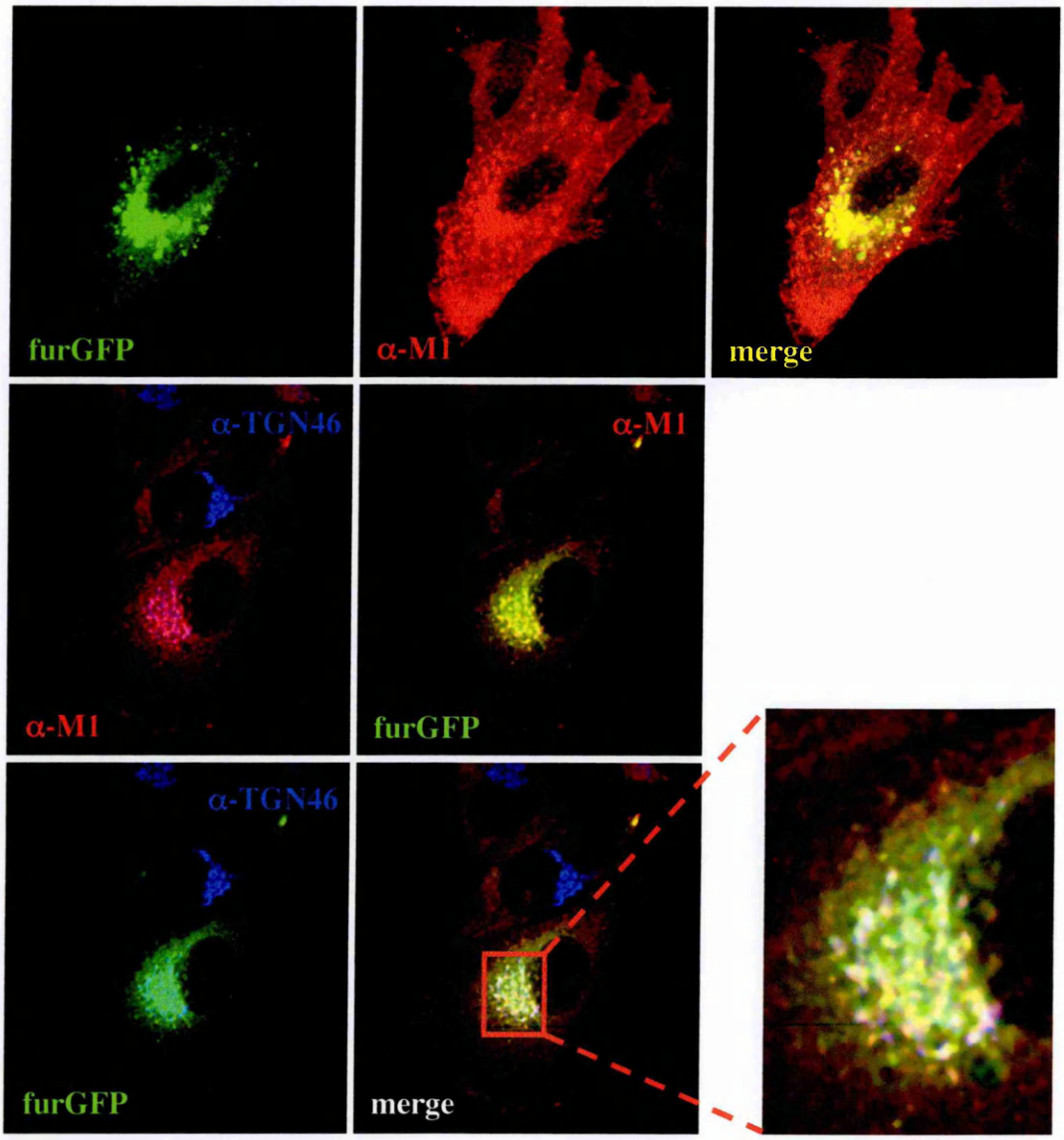


Fig. 4.12. Subcellular distribution of mature MT1-MMP in A375 melanoma cells by immunofluorescence. A375 cells co-transfected with F112-MT1-MMP and furin+GFP chimaera (FurGFP) were plated on coverlips for 16 hours, then fixed and analyzed at the confocal microscope as described in Material and Methods section. Cells were double-labeled with the $CaCl_2$-sensitive immunopurified monoclonal anti-FLAG antibody (M1) and with the sheep polyclonal antibody directed against TGN46. Combined stainings are shown with different combinations and the relative colours are also indicated. A region of interest of the merge staining is shown at a 10X magnification.

GFP-tagged Rabs. It is known that the GFP tag does not affect either localization or function of Rab proteins (Sonnichsen et al., 2000). Moreover, when overexpressed at low levels, most Rab proteins do not affect the morphology of the subcellular compartments (Weigert et al., 2004). Mature MT1-MMP was partially colocalized with Rab4 (not shown), but was completely divergent from Rab7 (Fig. 4.13 A), a protein involved in trafficking from early endosomes to late endosomes/lysosomes and localized in late endosomes (Press et al., 1998). Strikingly, mature MT1-MMP showed a distinct, highly overlapping pattern with Rab11 (Fig. 4.13 A), a protein localized to the recycling endosome, the TGN and specialized storage membranes of regulated secretory pathways (Ullrich et al., 1996).

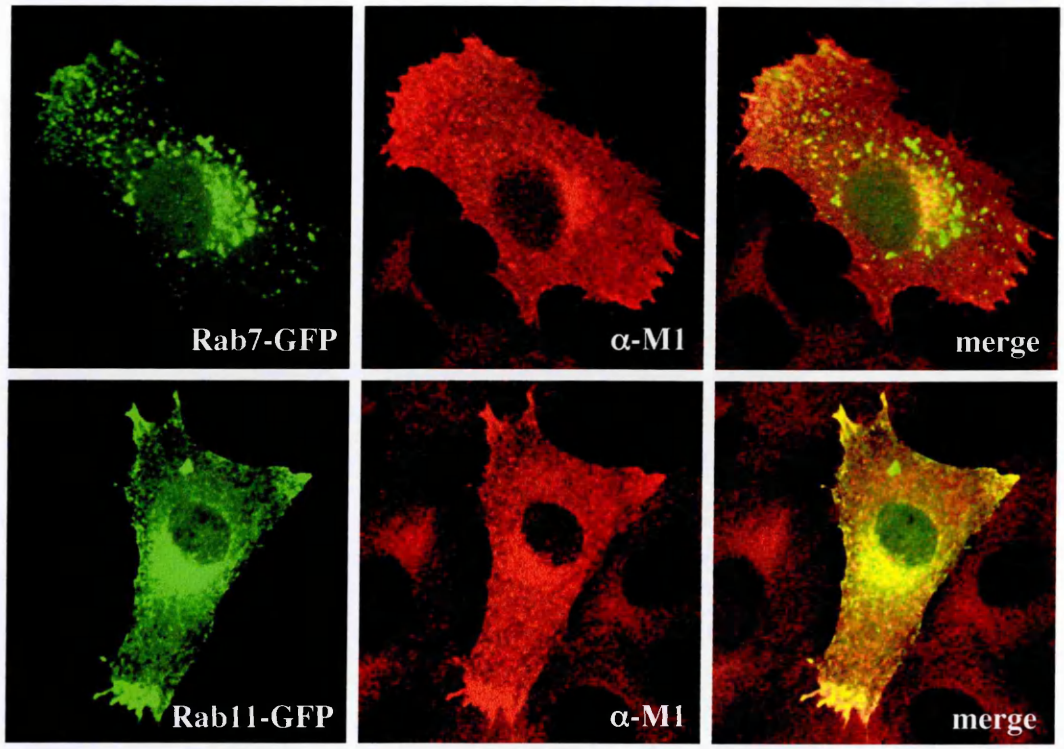
In conclusion, these data define: 1) the presence of mature MT1-MMP in association with endocytic membranes and, in particular, 2) the presence of mature MT1-MMP in a Rab11-positive compartment supporting the idea that MT1-MMP activation compartment is associated with recycling membranes, likely available for polarized/regulated delivery to the PM (e.g. GLUT-4, MHC-I compartments) (Fig. 4.13 B).

4.2.6 Co-immunoprecipitation between furin and proMT1-MMP

Pei and coworkers have recently found that a major portion of internalized MT1-MMPs are co-localized with furin, a protein "bicycling" between the trans-Golgi network and endosomes as well as between endosomes and the plasma membrane (Wang et al., 2004). In addition, they also found that the colocalization pattern between MT3-MMP and furin was observed even upon inhibiting furin activity or proMT3-MMP processing (Kang et al., 2002).

The interaction between furin and MT3-MMP was thus found to be relatively strong and resistant to a wide variety of treatments although a more detailed characterization was not performed. Since this tight interaction could be

A



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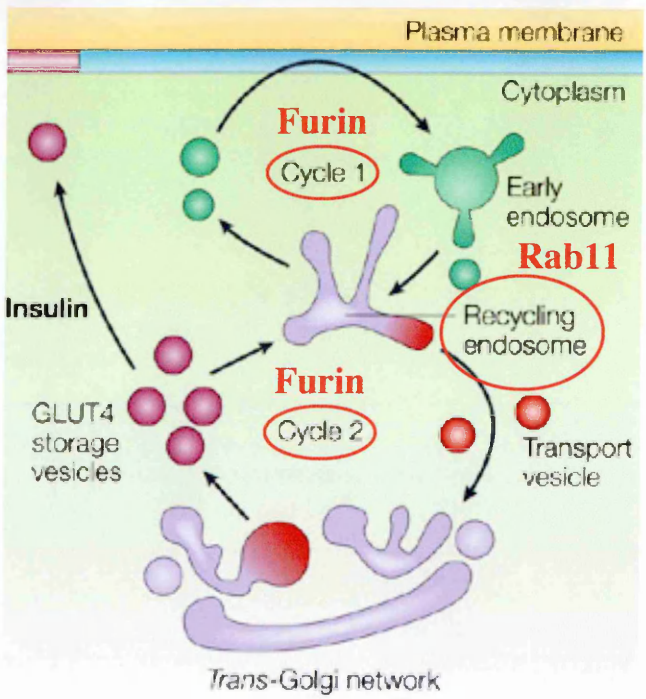

Fig. 4.13. Association of mature MT1-MMP with Rab11-positive recycling membranes. (A) A375 cells co-transfected with F112-MT1-MMP and Rab7-GFP or Rab11-GFP were plated on coverlips for 16 hours, then fixed and analyzed at the confocal microscope as described in Materials and Methods section. Cells were labeled with the $CaCl_2$-sensitive immunopurified monoclonal anti-FLAG antibody (M1). (B) A schematic representation of a hypothetical recycling compartment enriched with furin and Rab11 (modified from Maxfield et al., 2004).

common to other MT-MMPs, I decided to verify the interaction between furin and MT1-MMP by performing co-immunoprecipitation experiments. Furin-GFP (GFP-tagged at its C-terminal) and wild-type MT1-MMP were co-expressed in A375 melanoma cells. As shown in Fig. 4.14, MT1-MMP was co-immunoprecipitated with furin-GFP when I used a commercial polyclonal anti-GFP antibody (Abcam) but there was no co-immunoprecipitation when I used the anti-MT1-MMP antibody (MMR2). A possible explanation for this conflicting observation comes from the fact that the polyclonal anti-MT1-MMP antibody used in these experiments (MMR2) is raised against the propeptide-deleted extracellular region of MT1-MMP. Since it is likely that furin interacts with MT1-MMP within its extracellular region, it is possible that furin binding could interfere with the interaction between MT1-MMP and the MMR2 antibody, but not between the C-terminal GFP tag and the anti-GFP antibody.

Considering this evidence, at this level, I can only assume that MT1-MMP and furin form a complex via either direct or indirect interaction. Of note, furin appears to interact only with immature proMT1-MMP. For this reason, it could be hypothesized that this complex requires also the propeptide of MT1-MMP. The characterization of this interaction is now under investigation and I have preliminary indications that it has a functional role in the regulation of MT1-MMP activation by regulating MT1-MMP association with DRM (see section 4.3.3).

4.3 Discussion

4.3.1 Intracellular activation of MT1-MMP

Previous findings (Yana and Weiss, 2000) have suggested that MT1-MMP processing is dependent on a furin-like activity, while others have proposed that MT1-MMP does not require the cleavage of its propeptide to activate MMP2

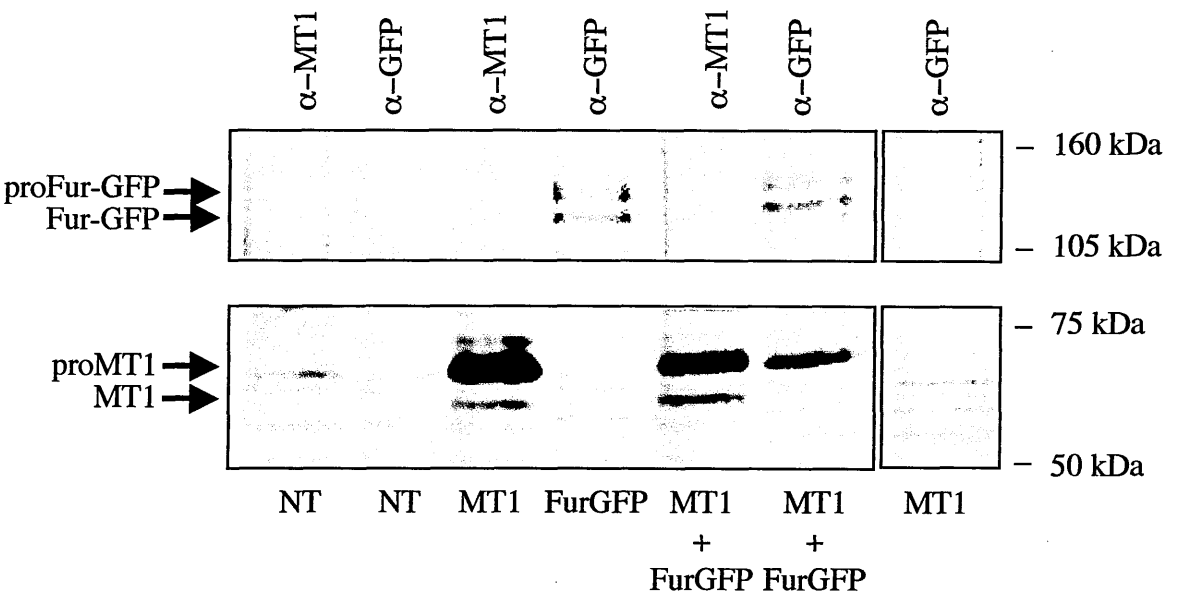

Fig. 4.14. ProMT1-MMP can be coimmunoprecipitated with FurinGFP. A375 melanoma cells untransfected (NT) or transfected with: MT1-MMP, FurinGFP or MT1-MMP+FurinGFP were labeled with [^{35}S]-methionine/cysteine for 2 hours and then lysed with RIPA buffer (150mM NaCl, 1%NP-40, 0.5% DOC, 0.1% SDS, 50mM Tris pH 8.0). As indicated, small amounts of cleared lysates from differently transfected cells were immunoprecipitated, as described in the Materials and Methods section, with a polyclonal immunopurified antibody raised against MT1-MMP, MMR2 (a-MT1) or with polyclonal immunopurified antibody raised against GFP (a-GFP). The black arrows mark the position of specific bands detected by autoradiography. The relative standard molecular weights are reported on the right.

in certain cell types (Cao et al., 1996). In addition, activation of MT1-MMP could also occur by plasmin at the cell surface (Okumura et al., 1997). To verify the activation mechanism in our experimental system, we co-transfected MT1-MMP with the furin inhibitor α1-PDX in A375 cells. In these conditions, MT1-MMP-dependent gelatin degradation was completely inhibited, indicating that MT1-MMP needs to be processed to express direct or indirect gelatinolytic activity. In addition, since in such conditions the mature 60 kDa form is undetectable, alternative pathways of activation, e.g. extracellular plasmin (Okumura et al., 1997) can be excluded. Finally, BB94, a broad-range metalloprotease inhibitor (Davies et al., 1993), did not affect MT1-MMP processing excluding that, at least in our conditions, MT1-MMP was functioning as a self-convertase as proposed by one group (Rozanov and Strongin, 2003). These data enhance the essential role of furin in the regulation of MT1-MMP processing and activity.

Despite the central role played by furin in many pathophysiological processes, the exact subcellular sites of processing and activation of its substrates remain elusive. This is also the case for MT1-MMP. Hence, a major concern is to identify the cellular location(s) where endoproteolytic cleavage of MT1-MMP occurs. A number of studies have established that furin is mainly localized in the trans-Golgi network but undergoes extensive cycling between the Golgi complex, endosome compartments and the plasma membrane (for a review, see Molloy et al., 1999). Of note, a high-resolution immunogold electron microscopy-based study has recently shown that, in addition to the Golgi apparatus, furin was clearly localized to endosomes and plasma membranes of a number of cell types (Mayer et al., 2004a). My finding that MT1-MMP processing takes place in an intracellular, post-Golgi, pre-plasma membrane compartment, are consistent with this widespread distribution of furin.

The extracellular face of the plasma membrane was recently proposed to be the MT1-MMP activation compartment solely based on experiments showing that, after chemical cross-linking, furin co-immunoprecipitates with proMT1-

MMP at the plasma membrane (Mayer et al., 2003). The same study also showed a colocalization of the two proteins on the plasma membrane. I show that, at least in A375 melanoma cells, furin-dependent processing of MT1-MMP occurs in a post-TGN, pre-plasma membrane compartment.

4.3.2 Morphological and functional characterization of the intracellular activation compartment of MT1-MMP

At steady-state, I find MT1-MMP to be mainly localized to vesiculo-tubular structures distal to the Golgi apparatus and close to the plasma membrane, possibly corresponding to the MT1-MMP activation compartment.

Since this compartment also contains recycling endosomal elements (e.g. Rab4, Rab11) and considering that I also find that MT1-MMP processing occurs during the exocytic process and not after re-entry through endocytic recycling, it appears that MT1-MMP is sorted to a recycling endocytic compartment where its maturation occurs. I am currently investigating the possibility that this is a transient storage compartment where MT1-MMP is available for rapid polarized/regulated trafficking to the plasma membrane. This hypothesis is particularly intriguing because pericellular matrix degradation during cancer invasion is dependent on activation of MMP2 by MT1-MMP. This activation has generally been considered to be a relatively slow process occurring as a result of enhanced expression of MT1-MMP. Cao and co-workers have recently reported that concanavalin A treatment of HT1080 fibrosarcoma cells is followed by MT1-MMP-induced activation of MMP2 on the cell surface within 1 hour. MT1-MMP function at the cell surface was also accelerated by treatment of cells with cytochalasin D, a drug which depolymerizes actin filaments, PMA, a stimulator of protein kinase C, and bafilomycin A, an inhibitor of lysosome/endosome function. A functional pool of intracellular MT1-MMP available for trafficking to the cell surface was demonstrated by repetitive ConA stimulation (Zucker et al., 2002).

These data further support my hypothesis that MT1-MMP is sorted to a transient storage compartment (trans-Golgi network/endosomes), where it is available for rapid trafficking to the plasma membrane and cell surface proteolytic activity.

4.3.3 A possibile role of furin in MT1-MMP intracellular trafficking

Furin, a low abundance protein present in multiple cell types, is a member of the proprotein convertase family that include Kex2, PC1/3, PC2, PC4, PACE4, PC5/6, and PC7. It is translated in the endoplasmic reticulum as an integral membrane precursor prior to activation (for a review, see Thomas, 2002). Autoproteolysis cleaves an N-terminal peptide that binds, in part, to the catalytic domain and inhibits enzymatic activity. Once translocated to the Golgi stack, this peptide is cut and released to produce fully active furin. Although, at steady state, furin is primarily (>80%) localized to the trans-Golgi by a signal on its carboxyl cytoplasmic tail, a significant amount of furin cycles between the Golgi and the plasma membrane and small amounts have been detected in the extracellular space. Thus, even though furin is available to cleave MT-MMPs, inhibition of the furin catalytic activity as well as mutation of the furin binding site in proMT3-MMP does not prevent the intracellular trafficking, the posttranslational processing, or the furin colocalization of MT3-MMP (Kang et al., 2002).

These unexpected results are consistent with my observation of a co-immunoprecipitation of proMT1-MMP with furin. The physical features of this interaction (direct or indirect) as well as the exact station of the secretory pathway where this interaction would occur are still not defined and are beyond the scope of my thesis. At any rate, I can speculate that proMT-MMPs traffic through the trans-Golgi and progress to the plasma membrane via a furin-regulated pathway, either separately or complexed with regulatory proteins. Exposure to environmental changes (pH, ions, etc.) could result in conformational changes that trigger cleavage of the propeptide domain by a membrane-anchored furin within a post-trans-Golgi network compartment to facilitate subsequent MT1-MMP cell-

surface presentation and activity. Together with membrane partitioning, my data identify furin as another possible regulator of MT1-MMP-dependent activities.

Of note, I recently found that MT1-MMP distribution profile within different membrane subdomains from A375 melanoma is considerably altered when furin is overexpressed (Fig. 4.15 A). This effect seems to be dependent on furin expression levels and indeed did not occur when I cotransfected the specific furin inhibitor α1-PDX.

Taken together, I found that furin is responsible for intracellular pro-MT1-MMP activation and, at least in A375 cells, is associated only with detergent-soluble membranes and co-immunoprecipitates only with proMT1-MMP. Considering this, my working hyphothesis is now that furin could be a possible molecular "bait" for proMT1-MMP, regulating its partitioning with different membrane subdomains and therfore its biochemical processing and biological activities (Fig. 4.15 B).

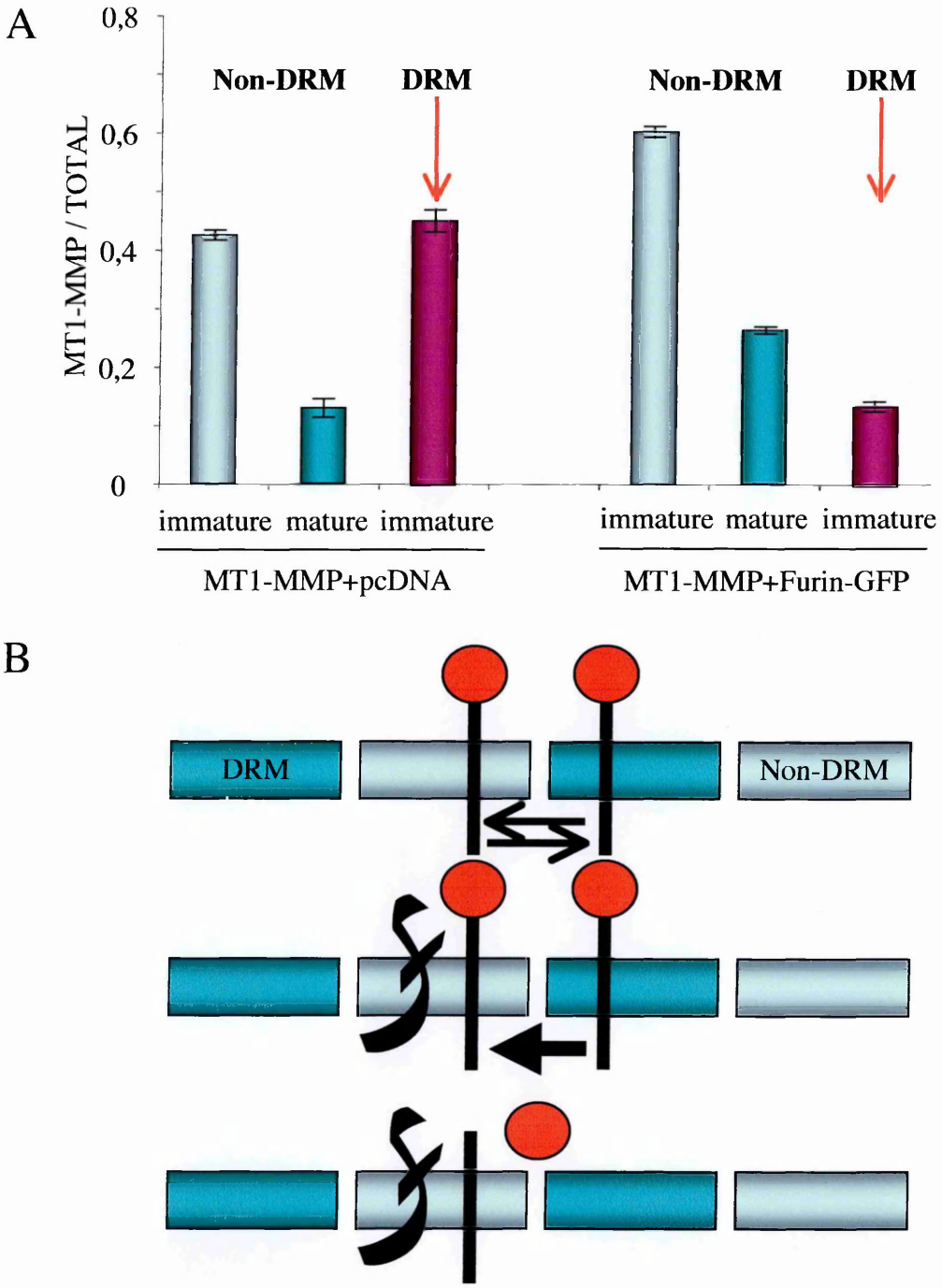

Fig. 4.15. MT1-MMP membrane partitioning depends on furin expression levels. (A) A375 cells transfected with MT1-MMP and pcDNA, or with MT1-MMP and Furin-GFP were labeled with [^{35}S]methionine/cysteine for 2 hours and lysed with TNE buffer containing 1% Triton X-100 on ice. Soluble (S) and insoluble (P) fractions were separated by ultracentrifugation, immunoprecipitated with a polyclonal anti-MT1-MMP antibody and analyzed by SDS-PAGE/autoradiography. The processing ratio of MT1-MMP (MT1-MMP/Total) was determined with the Fuji BAS software and plotted graphically. (B) Proposed model for the role of furin on MT1-MMP membrane partitioning and activation. Legend: DRM (detergent-resistant membranes), F (furin). The propeptide domain of MT1-MMP (black stick) is shown in red.

CHAPTER 5

Final Discussion

5.1 Intracellular Processing and Activation of MT1-MMP Depends on its Partitioning into Lipid Domains

It has clearly emerged that MT1-MMP (and perhaps other membrane-type MMPs) is essential for the growth of human cancers, and that it acts by disrupting the three-dimensional matrix which would otherwise impede cell proliferation (Hotary et al., 2003) (Fig. 5.1). Hence, full understanding of MT1-MMP physiology is crucial for developing strategies aimed at preventing the escape of cells from a primary tumor. In the present study I show that only a subpopulation of MT1-MMP, associated with detergent soluble membranes, is activated and rapidly degraded. In contrast, DRM-associated MT1-MMP retains its propeptide and is stably linked with the plasma membrane. These findings establish a novel mode of regulation of MT1-MMP activity and suggest a potential molecular basis for its diverse functions in cancer progression (see Fig. 1.7 from Introduction).

The results obtained by combining biochemical experiments with an in-depth morphological analysis indicate that the metabolic fate of proMT1-MMP is dependent on its partitioning into different membrane domains. In particular, furin-dependent activation, shown here to occur in a post-Golgi, pre-plasma membrane compartment, occurs exclusively in the detergent-soluble fraction (Fig. 5.2). Also, this processed MT1-MMP is the sole form responsible for ECM degradation in vitro.

The functional role of DRM-associated MT1-MMP remains to be unveiled. A potential indication stems from the findings that wild-type

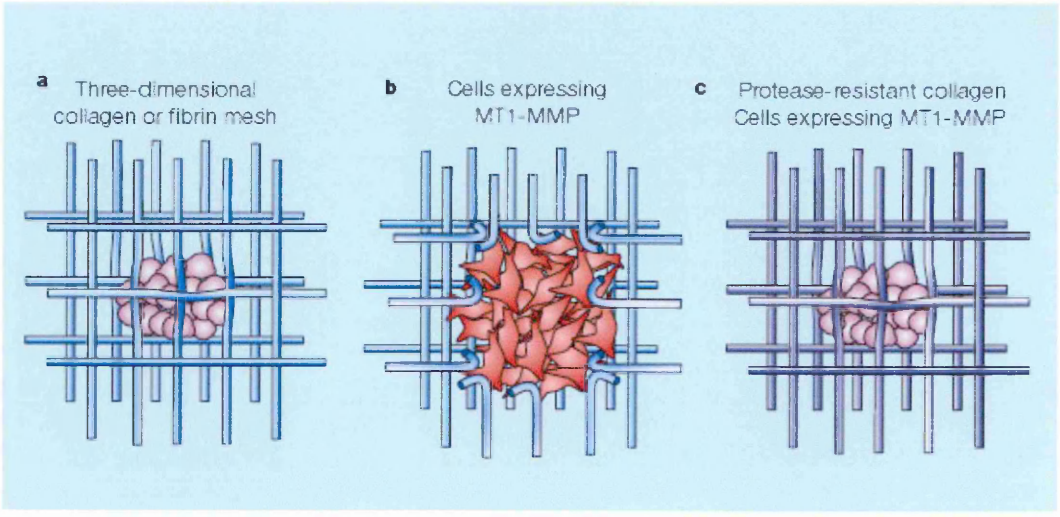

Fig. 5.1. MT1-MMP usurps tumor growth control imposed by the three-dimensional extracellular matrix (reproduced from Hotary et al., 2003). Cancer cells are able to proliferate at accelerated rates within the confines of a three-dimensional (3D) extracellular matrix (ECM) that is rich in type I collagen. The mechanisms used by tumor cells to circumvent endogenous antigrowth signals have yet to be clearly defined. We find that the matrix metalloproteinase, MT1-MMP, confers tumor cells with a distinct 3D growth advantage in vitro and in vivo. The replicative advantage conferred by MT1-MMP requires pericellular proteolysis of the ECM, as proliferation is fully suppressed when tumor cells are suspended in 3D gels of protease-resistant collagen. In the absence of proteolysis, tumor cells embedded in physiologically relevant ECM matrices are trapped in a compact, spherical configuration and unable to undergo changes in cell shape or cytoskeletal reorganization required for 3D growth. These observations identify MT1-MMP as a tumor-derived growth factor that regulates proliferation by controlling cell geometry within the confines of the 3D ECM.

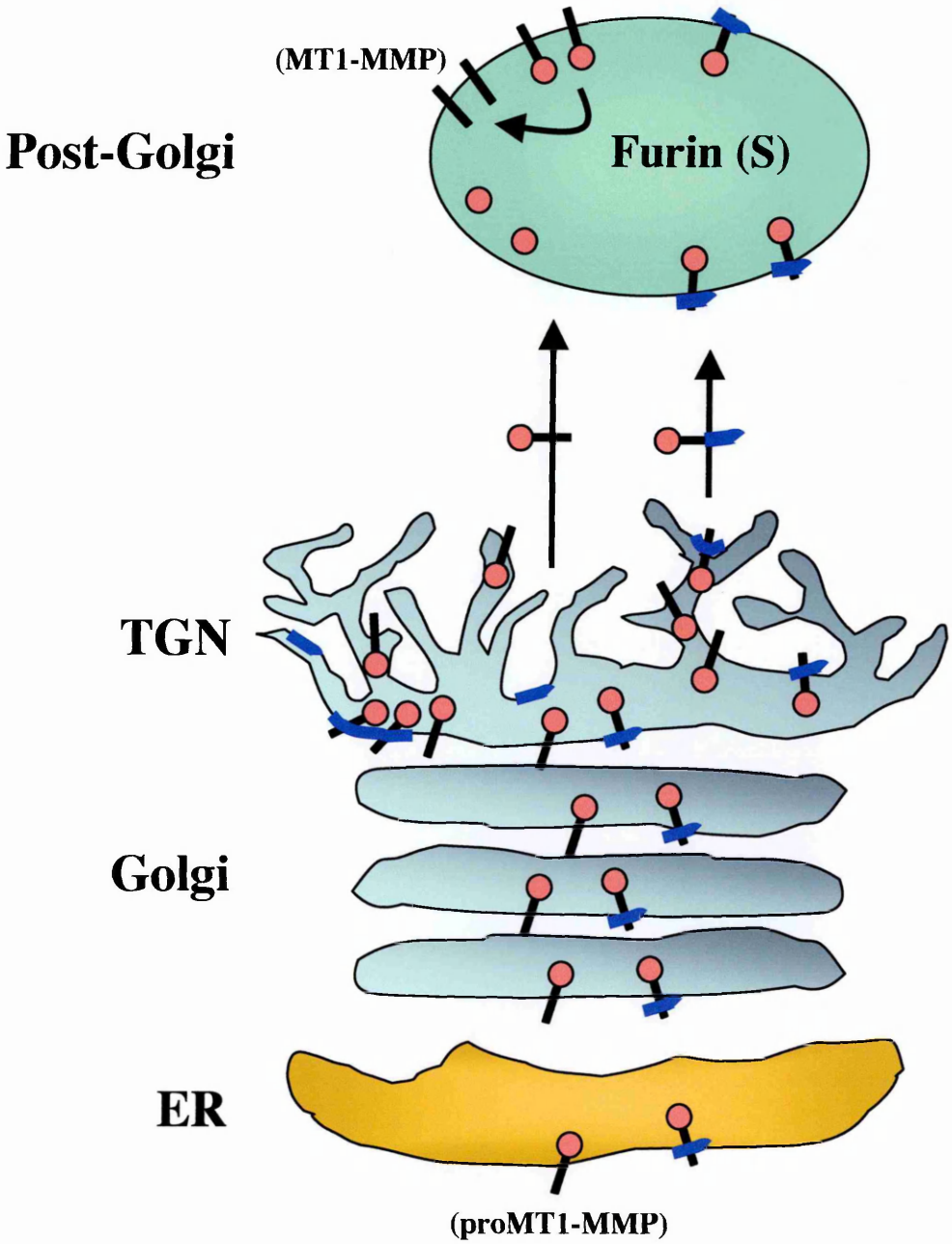

Fig. 5.2. Proposed model: Intracellular Processing and Activation of MT1-MMP Depends on its Partitioning into Lipid Domains. The majority (80%) of MT1-MMP is sorted to detergent-resistant membrane fractions (blue); however, it is only the minor (20%) detergent-soluble fraction of MT1-MMP that undergoes intracellular processing to the mature form. Also, this processed MT1-MMP is the sole form responsible for ECM degradation in vitro. Finally, furin-dependent processing of MT1-MMP is shown to occur intracellularly after exit from the Golgi apparatus and prior to arrival at the plasma membrane, in a post-Golgi compartment. Of note, in this model, furin is exclusively associated with detergent-soluble membranes (S). The association of MT1-MMP with different membrane subdomains might be crucial in the control of its different activities: for instance in cell migration and invasion and other less defined ones such as MT1-MMP-dependent signaling pathways.

MT1-MMP overexpression triggers different signaling pathways (see next section). Since DRM domains represent a preferred platform for a number of signaling receptors and cytoskeleton interactors (Toomre et al., 2000), a possibility that remains to be tested is whether the DRM-associated proMT1-MMP pool that I characterized in this work may be involved in the transduction of intracellular signal pathways.

5.2 DRM-associated MT1-MMP may induce intracellular signal transduction via its cytoplasmic domain

A number of recent findings support the idea that the localization of plasma membrane MT1-MMP in DRM, specialized membrane domains rich in receptors and signaling intermediates, could induce signal transduction and regulate gene expression via its cytoplasmic domain.

The cytoplasmic domain of MT1-MMP is distinct from those of MT2-, MT3-, and MT5-MMP, and is well characterized. In addition to playing an important role in MT1-MMP localization and proMMP-2 activation through homophilic complex formation at the cell surface (see section 1.8.3), the cytoplasmic domain of MT1-MMP could participate in the following cell signaling events.

In cells transfected with MT1-MMP, the cytoplasmic domain of MT1-MMP is involved with the activation of the extracellular signal-regulated protein kinase (ERK) cascade during cell migration (Gingras et al., 2001). More recently, Takino *et al.* have demonstrated that MT1-MMP-mediated ERK activation leads to tumor cell invasion in type-1 collagen gel (Takino et al., 2004).

The cytoplasmic tail of MT1-MMP is also required for the activation of the Src-tyrosine kinase pathway involved with VEGF up-regulation during tumoral angiogenesis (Sounni et al., 2004). More recently, Uekita *et al.* have identified a new 19 kDa MT1-MMP cytoplasmic tail binding protein-1 (MTCBP-

1), by yeast two-hybrid screening. This protein, homologue to members of cupin superfamily, is distributed between three subcellular compartments (membrane, cytoplasm and nucleus) and may inhibit the invasion and migration-promoting activities of MT1-MMP (Uekita et al., 2004).

Interestingly, it has been recently described that hyaluronic acid (HA) cell surface binding to its receptor, the membrane glycoprotein CD44, is inhibited by the overexpression of full-length MT1-MMP in endogenously MT1-MMP-expressing tumor cells (Annabi et al., 2004). This inhibition is dependent on the cytoplasmic domain of MT1-MMP and may be overcome by shifting part of the MT1-MMP pool out of DRMs by cholesterol extraction in presence of collagen (Annabi et al., 2004). This evidence strongly supports my idea that DRM-associated proMT1-MMP pool may induce intracellular signalling pathways inhibiting cell functions associated with an increased CD44-mediated HA binding (e.g. tumor cell migration). For this reason, I will discuss this topic in further detail in the following section.

5.3 DRM-associated MT1-MMP may regulate CD44-mediated tumor cell migration

MT1-MMP is a multifunctional protein that regulates several biological processes at the cell surface of cancer cells (i.e. cell migration). As already mentioned (Annabi et al., 2004), it has been recently proposed that MT1-MMP regulates increased CD44-mediated HA binding of some tumor cells (e.g. highly invasive glioma cells). HA is an important glycosaminoglycan ECM constituent believed to be implicated in angiogenesis, the formation of new blood vessels from preexisting vasculature. Although the serum level of HA is already used as an indicator of progressive malignant disease, its effects on *in vivo* angiogenesis and endothelial cell (EC) function are complex and have been reported to depend on HA concentration and molecular size. Accordingly, although high molecular

weight HA was shown to inhibit EC functions, low molecular weight HA stimulated EC proliferation, tubulogenesis, and neovascularization (for a recent review, Adamia et al., 2005). Moreover, small HA polymers efficiently regulated CD44 cell surface functional expression and thus promoted CD44-dependent tumor cell migration. In fact, elegant studies demonstrated that CD44 directed MT1-MMP to lamellipodia by associating with its hemopexin-like domain, and that cell surface MT1-MMP-mediated cleavage of CD44 subsequently played a critical role in promoting tumor cell migration (Kajita et al., 2001; Mori et al., 2002).

More recently, Beliveau *et al.* demonstrated that MT1-MMP overexpression inhibits CD44-mediated HA binding through a ERK-dependent pathway, and this effect is antagonized by MMP inhibitors when collagen is present (Annabi et al., 2004). In this process, ECM components are also involved. Type I collagen, for example, not only triggers MT1-MMP-dependent proMMP-2 activation through the formation of a trimolecular complex involving TIMP-2 (Fig. 5.3), but also induces a profound cytoskeletal reorganization that signals an increase in HA cell surface binding to CD44.

Although the cytoplasmic domain of MT1-MMP is unnecessary for proMMP-2 processing, it is crucial for MT1-MMP cell migration and activation of the MAPK pathway, as well as CD44 cell surface functional expression. Down-regulation of HA binding activity by inhibition of the MAPK cascade further suggests that an MT1-MMP/CD44/ERK-dependent regulatory signaling regulates HA cell surface binding in tumor cells (Annabi et al., 2004).

Cholesterol depletion also increases the cell surface binding of HA in synergy with type I collagen (Annabi et al., 2004). Cholesterol depletion may trigger a further increase in type I collagen interaction with caveolae-associated proteins, such as DRM-associated proMT1-MMP, but this needs to be confirmed.

This study suggests that DRMs represent a new modality in regulating the functional activity of MT1-MMP in malignant cells, and that the yet-to-be

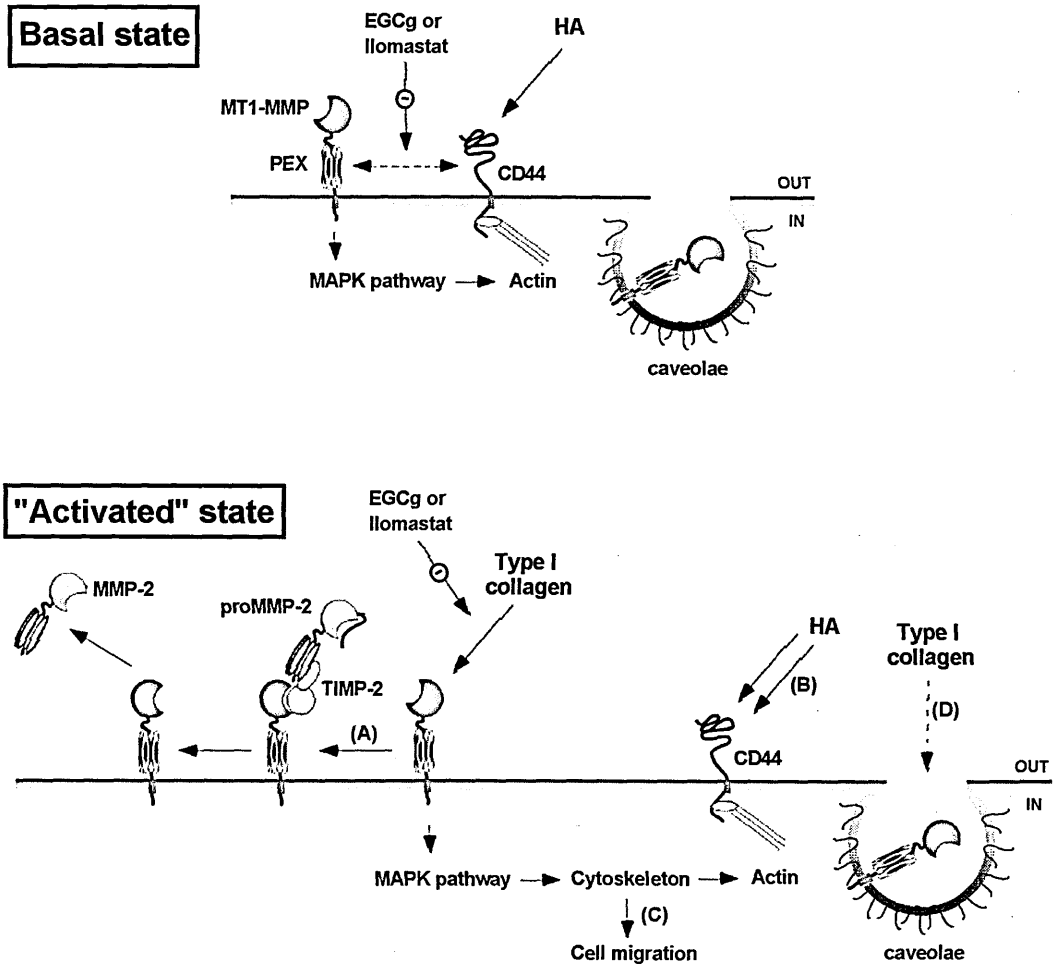

Fig. 5.3. MT1-MMP and its caveolar location may regulate the CD44-mediated HA cell surface binding in tumor cells (reproduced from Annabi et al., 2004). In the basal state, MT1-MMP regulates CD44 cell surface expression through a MAPK-dependent pathway, and this is antagonized by two inhibitors of MMP functions: Ilomastat and EGCg, a green tea catechin with anticancer and anti-angiogenic properties. In the "activated" state, type I collagen triggers proMMP-2 activation through the formation of a trimolecular complex involving TIMP-2, which subsequently releases an active form of MMP-2 (*A*). The profound cytoskeletal reorganization induced by type I collagen signals an increase in HA cell surface binding to CD44 (*double arrow, B*). Although the intracellular domain of MT1-MMP is unnecessary for proMMP-2 processing, it was crucial for MT1-MMP cell migration and activation of the MAPK pathway, as well as CD44 cell surface functional expression (*C*). Down-regulation of HA binding activity by inhibition of the MAPK cascade further suggests that an MT1-MMP/CD44/ERK-dependent regulatory signaling regulates HA cell surface binding in tumor cells. Cholesterol depletion may also trigger a further increase in type I collagen interaction with caveolae-associated proteins such as caveolar MT1-MMP (*D*), but this needs to be confirmed.

identified intracellular signal transduction pathways involving the cytoplasmic tail peptide sequence are likely to regulate DRM-associated proMT1-MMP-dependent activities.

Overall, these data, taken together with the data we recently published (Mazzone et al., 2004), provide new insight into the regulation of the metastatic processes based on the interaction of tumor cells with their ECM environment.

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